

STATEMENT

ANALYSIS OF BARLEY GENOMES - A MOLECULAR APPROACH

THE RESULTS DESCRIBED IN THIS THESIS WERE OBTAINED
BY MYSELF UNDER THE SUPERVISION OF DRS. COLIN H. DRY AND
H. C. SUBRAMANYAM.

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A THESIS SUBMITTED FOR THE DEGREE OF DOCTOR OF
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ABSTRACT

DNA's of three closely related diploid species of *Hordeum* (*H. vulgare*, *H. agriocrithon* and *H. spontaneum*) were compared among themselves and with a group of three species (*H. californicum* (2x), *H. jubatum* (4x) and *H. arizonicum* (6x)) related to each other in having one genome in common and forming a polyploid series.

Several types of bacteria were found to co-exist with different species of *Hordeum* and a method to sterilize seeds was introduced. As prepared from shoots, the maximum contribution of bacterial DNA in the total DNA preparation was estimated to be no more than 0.003% and at this level it did not interfere with the interpretation of results of DNA analysis from six *Hordeum* species.

Buoyant densities of the DNA's from these species fall within a narrow range (1.700-1.701 g/cm³). Melting temperatures (T_ms) of the DNA's are also similar (85.2 C-86.2 C). None of the DNA's show any satellite band in a neutral CsCl density gradient.

In Ag⁺-Cs₂SO₄ gradients DNA's of all the species resolved cryptic satellites, on both the lighter and heavier sides of the main band. The diploid series have similar, though not necessarily identical, satellite patterns. The members of the polyploid series show similar satellite patterns among themselves but distinct from those of the diploid series. In the polyploid group the amount of satellite shows a progressive decrease with increase of ploidy.

DNA's of *H. vulgare* and *H. arizonicum* were studied in more detail. The amount of repeated DNA (reassociating up to a C₀t of 100) in *H. vulgare* is 70% and in *H. arizonicum* 65% of the total. The C₀t curves of these two species are different especially at low C₀t values. The low C₀t fractions of *H. arizonicum* DNA contain components of low buoyant densities which are not present in the corresponding C₀t fractions of

H. vulgare DNA.

Three heavy and two light satellites from *H. vulgare* DNA and one heavy and two light satellites from *H. arizonicum* DNA were purified by preparative ultracentrifugation in $\text{Ag}^+ - \text{Cs}_2\text{SO}_4$ gradients. The native satellite DNAs were analysed in terms of buoyant densities and thermal denaturation properties, as were the reassociated satellites. Some satellites were homogeneous by the criteria of buoyant densities, however, heterogeneity of each of the satellites was evident when the thermal denaturation data were subjected to differential analysis.

The H3 satellite of *H. vulgare* has two thermal components ($T_m = 83^\circ\text{C}$ and 91.5°C). The H1 of *H. arizonicum* has similar thermal components ($T_m = 82.5^\circ\text{C}$ and 91°C) plus an extra component ($T_m = 87^\circ\text{C}$) which makes this satellite distinct from the H3 of *H. vulgare*.

Radioactive RNAs complementary to the three heavy satellites of *H. vulgare* were localized by *in situ* hybridization to the nuclei and chromosomes of the same species. The H1 satellite of *H. arizonicum* was similarly localized to the nuclei and chromosomes of *H. arizonicum*. The latter satellite hybridizes well with the chromosomes of *H. californicum* but poorly with the other species tested. The H3 satellite, on the other hand, hybridizes well with the chromosomes of *H. spontaneum*, *H. agriocrithon*, *H. californicum* and *H. arizonicum*, suggesting that sequences similar, though not necessarily identical, to the H3 satellite are present in these species.

The DNA analysis data agree well with the existing taxonomic relationships among these six species.

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CHAPTER 1

INTRODUCTION

Traditionally the classification of plants is based mainly on morphological characters. In a phylogenetic study an attempt is made to establish the paths of evolution of the members of a particular species group in terms of the relationships between them. The latter takes into account fossil and archaeological records as well as the characteristics of the present day forms. Nowadays, besides morphology and development, evidence from protein analysis, secondary products (such as alkaloids, cytochrome c etc., reviewed by Turner, 1969) and cytogenetic analysis are also considered when establishing relatedness and divergence among different species. Cytogenetic analyses mainly utilize chromosome number and their morphology (karyotype), DNA content per cell and meiotic behaviour of chromosome in interspecific crosses.

An alternative approach to determine phylogenetic relationships is the analysis of DNA sequences themselves, since morphology, adaptation, differentiation and all other facets of an organism are, in the last analysis, the expression of genetic material, i.e., the DNA contained in the cell. This is a more direct way of examining the genome than indirect comparisons involving phenotypes. In molecular terms the genome may be defined as the quantity and quality of DNA in a haploid cell. In eukaryotic organisms the DNA, in association with proteins, is organized into a series of two or more chromosomes. With the exception of some fungi (Timberlake, 1978) this DNA consists of at least three types of sequences - unique, moderately and highly repeated (Britten and Kohne, 1968; Kohne, 1970). The unique sequences, which are present in single or very few copies, include the sites of classical genetic activity, i.e. the structural gene loci, though not all unique DNA appears to function in this way (Davidson *et al.*, 1977). Intermediate repeated sequences are present in large numbers and are interspersed with

the unique sequences. Depending on interspersion, two general types or organization are recognized:

- (i) Repetitive sequences of about 300 base pairs (bp) are interspersed with unique sequences of about 1000 bp (e.g. *Xenopus*, Britten, 1972; Davidson *et al.*, 1973; *Nicotiana*, Zimmerman and Goldberg, 1977; *Triticum*, Flavell and Smith, 1976; *secale*, Smith and Flavell, 1977; *Petroselinum*, Kiper and Herzfeld, 1978).
- (ii) Repetitive sequences of about 5600 bp in length are interspersed with unique sequences of about 13000 bp; e.g. *Drosophila*, (Manning *et al.*, 1975); *Chironomus*, (Wells *et al.*, 1976).

Highly repeated DNA sequences involve small stretches of DNA (2-12 bp long) repeated thousands to millions of times.

The importance of DNA analysis in phylogenetic and evolutionary studies has been recognized for over fifteen years (McCarthy and Bolton, 1963; Hoyer *et al.*, 1964; Bendich and Bolton, 1967; Kohne, 1968; Turner, 1969; Bendich and McCarthy, 1970a, 1970b; Nagl and Capesius, 1976; Belford and Thompson, 1976). There is, however, a lack of comparative data, especially for plant DNA, and many more studies are required before meaningful conclusions can be drawn. The few reports towards this approach have been mainly at the generic level or have involved higher taxonomic categories (Bendich and Bolton, 1967; Bendich and McCarthy, 1970a; Flavell *et al.*, 1977). DNA comparisons of different species within the same genus are available in wheat (Bendich and McCarthy, 1970b; Mitra and Bhatia, 1972); *Vicia*, (Chooi, 1971); *Phaseolus*, (Beridze, 1972); *Brassica* (Beridze, 1975); *Osmunda*, (Stein and Thompson, 1975; Belford and Thompson, 1976); *Gossypium*, (Wilson *et al.*, 1976); *Neurospora*, (Dutta *et al.*, 1976); *Zea*,

(Levings, II *et al.*, 1976); *Lathyrus*, (Narayan and Reese, 1977); *Allium* (Ranjekar *et al.*, 1978b) and *Hordeum*, (Chakrabarti *et al.*, 1978b, a preliminary report of part of this thesis). The evidence used in such comparisons has, in the main, been of a fragmentary nature; for example, Beridze (1972, 1975) used only buoyant density analysis in CsCl gradients. Therefore no real opportunity exists to provide a complete picture of the molecular consequences of speciation.

Buoyant density and melting temperature are two of the most easily obtained characteristics of DNA. These two parameters do not seem to differ much among different species within the same genus (Dutta *et al.*, 1976; Levings, III *et al.*, 1976; Subrahmanyam and Azad, 1978; Ranjekar *et al.*, 1978b; Chakrabarti *et al.*, 1978b). Similarities between buoyant densities and melting temperatures within a genus reflect the similarity of the base composition of their DNA. From the evolutionary viewpoint, therefore, these characteristics, in themselves, are only of limited value and require supplementation with other data.

One of the main tools for phylogenetic studies at the molecular level is DNA/DNA hybridization. This provides a relatively crude means for detecting the DNA sequences held in common between two organisms. The two strands of DNA can be separated by raising the temperature (thermal denaturation) and these separated strands can then be allowed to reassociate, at the appropriate temperature and salt concentration, to form a duplex. During such reassociation the separated strands of one species can react with those of another, provided there is homology in base sequences. The degree of hybridization and the thermal stability of the hybrid DNA so obtained provides a measure of the relatedness of the two species in question.

This approach has already been applied to several species of related and unrelated plants (Bendich and Bolton, 1967) as well as to four species of *Gramineae* (Bendich and McCarthy, 1970a; Smith and Flavell, 1974), four biotypes of wheat (Bendich and McCarthy, 1970b), six species of *Vicia* (Chooi, 1971), three species of *Osmunda* (Stein and Thompson, 1975), and four species of *Atriplex* (Strong and Thompson, 1975). In the *Graminea*, the results of such DNA hybridization supported the conventional classification, whereas those from *Osmunda* and *Atriplex* have given a different picture from that obtained using conventional taxonomic approaches. One needs to bear in mind that it is only the repeated fraction of the DNA which would react in such hybridization experiments. More recently, techniques have been developed which make it possible to separate the repeated fractions of the DNA from the unique sequences and study them independently. DNA/DNA hybridization of repeated sequences in barley, oat, rye and wheat have also confirmed the phylogenetic relationships that have been derived from morphological analysis and have also shown that the amount of repeated fractions held in common diminishes with taxonomic separation of the species (Flavell et al., 1977). Additionally this study revealed that each species has its own type of repeated sequences which appears to have arisen after the species diverged from a common ancestor. The hypothesis that repetitive DNA arose during a short period of evolutionary time (Walker, 1971) and that single copy DNA (unique sequence) held a better 'palaeontological record' (Kohne, 1970) than repetitive DNA has led several investigators to prefer the use of single copy DNA for phylogenetic analysis (Angerer et al., 1976; Rice and Paul, 1971; Belford and Thompson, 1976). Thus contrary to the evidence from DNA/DNA hybridization using repetitive DNA, an equivalent study involving single copy DNA among four species of *Atriplex* gave support to the traditional concept of the phylogenetic relationships between them (Belford and Thompson, 1976).

In many instances a particular class(es) (definiable by buoyant density) of highly repeated DNA can be isolated in pure form (see below). Chapters III and IV of this thesis deal with this class of highly repeated DNA. In order to give the reader some understanding of the techniques and assumptions involved in using DNA analysis to determine interrelationships between a series of species, it will be helpful at this point to summarise what is currently known about the properties of highly repeated DNA within the genome.

1. *Satellite DNA*

In many cases the pattern of repetition of a given sequence (at least one hundred thousand tandem repeats, Salser *et al.*, 1976) results in this fraction of DNA banding in a CsCl gradient at a different density to the bulk of the DNA (main band). The additional band(s) is referred to as a satellite band in contrast to the main band DNA. In some cases a satellite band(s) can be observed only after binding of DNA with antibiotics (such as actinomycin D, Dunsmuir, 1976; Dennis *et al.*, 1979) or heavy metals (for example, Hg^{2+} and Ag^+ , Corneo *et al.*, 1968, 1970; Nandi *et al.*, 1965; Ranjekar *et al.*, 1976; Timmis *et al.*, 1975; Filipski *et al.*, 1973) followed by centrifugation in a CsCl or Cs_2SO_4 gradient respectively. Satellites of this type are called cryptic satellites. However, the absence of satellite DNA in a density gradient does not necessarily mean the lack of highly repeated DNA sequences in a genome.

A great deal is now known about satellite DNA from animal genomes (reviewed by Walker, 1971; Bostock, 1971; Rae, 1972; Appels and Peacock, 1978; John and Miklos, 1979). By contrast, the information about the satellite DNA component of plant genomes is much more limited and therefore it is not possible, at present, to infer whether DNA of plants and animals share common characteristics.

Amongst the plants that have been studied, monocotyledons in general do not show satellite bands in a CsCl density gradient whereas many dicotyledonous species have such density satellites (reviewed by Ingle et al., 1973, 1975; Nagl, 1976; Coudray et al., 1970). The orchid *Cymbidium* is the only monocot which shows a satellite in a CsCl gradient (Capesius et al., 1975). Cryptic satellites in monocots have, however, been observed in barley (Ranjekar et al., 1976; Deumling et al., 1976; Peacock et al., 1977a; Chakrabarti et al., 1978b), wheat (Huguet and Jouanin, 1972, Ranjekar et al., 1976; Deumling et al., 1976; Peacock et al., 1977a, 1977b), rye (Appels et al., 1978) and in *Scilla* (Timmis et al., 1975).

2. *Species Specificity of Sattelite*

When centrifuged in an isopycnic density gradient, related species often show a distinctive satellite pattern. That is, the satellite pattern of one species differs from that of another, in kind (*Ammospermophilus harrisi* - *Mus musculus*), amount (*Dipodomys ordii* - *D. agilis*) or both (*Drosophila virilis* - *D. americana*). Comparable differences of satellite pattern among related species, even species within the same genus, are also found in plants (Ingle et al., 1973; Beridze, 1972, 1975; Chapter III and IV of this thesis).

3. *Sequence Similarity within a Species*

When there is more than one satellite in a species, as shown by different physical properties, such as buoyant density and melting temperature, they may be similar to each other in sequence arrangement. In *Drosophila virilis*, for example, the three satellites are composed of a basic repeating unit of 7 base pairs and are related by one or two base changes (Gall and Atherton, 1974). In *D. melanogaster*, three of the four known satellites are related by single base substitutions in,

and rearrangements of, the basic di- and tri-nucleotide units (AA-)m-(A-)n, but unlike *D. virilis*, more than one sequence can occur within one satellite DNA band (Brutlag and Peacock, 1975; Endow et al., 1975). The 1.688 satellite of *D. melanogaster*, however, is much more complex than this. The situation in *Dipodomys ordii* (Salser et al., 1976) is different from that of *Drosophila*. Satellite DNAs in the former are not similar, or related, in their sequences. Therefore, the generalized claim of sequence similarity within a species is not well founded.

In plants, only barley (Ranjekar et al., 1976; Chapters III and IV of this thesis) and cucumber (Kadouri et al., 1975; Timmis and Ingle, 1977) are known to contain more than one density satellite, but no data is yet available about their sequences. Therefore, it is not possible to draw any conclusion regarding sequence similarity of satellite within any species of plants.

4. The Question of Conservation

There are claims that satellite sequences are conserved in a broad evolutionary sense. Thus some repeated sequences or satellites may be present not only among species within a genus (*D. virilis* - *D. americana*, Gall and Atherton, 1974; *D. similans* - *D. melanogaster*, Lohe, 1977; *Gecarcinus lateralis* - and seven other species of crabs, Graham and Skinner, 1973) but also in different genera (*Macropus robustus robustus* - several members of the macropods group, Venolia, 1977; *Macropus refogresius* - *Megaleia rufa*, Dunsmuir, 1976; *Dipodomys* - Guinea-pig, Salser et al., 1976; Human-Chimpanzee, Prosser et al., 1973; wheat-barley, Peacock et al., 1977a). These conclusions are based on one or more of the following criteria: similarity between homologous and heterologous DNA/cRNA hybridization as well as thermal stability of the hybrid molecules, *in situ* hybridization and restriction

endonuclease analysis. It has been suggested that related species share a common 'library' of satellites (Salser et al., 1976). Some or all of this library may then be present in a species. If the same sequence(s) is amplified in two species then an identical satellite would result in each species.

It should be realized that the claims for conservation on the basis of similar physical properties and positive *in situ* hybridization are not convincing since these techniques are not sensitive enough to detect minor differences in satellite sequences. It is true that this approach may show that the satellites of two species are similar but conservation by definition demands absolutely identical sequences in the satellites concerned. This point can only be settled by sequence analysis which has been done only for a few animal satellite DNAs (see Table I, Chapter VI).

Most probably, conservation of satellite sequences is not a general phenomenon in the eukaryotic genome.

Although there is some evidence for conservation of sequence in related species, the amount of satellite is certainly not conserved. Amongst different species of plants within the same genus, the amount of satellite DNA may vary considerably (Beridze, 1972, 1975). At one extreme in *Brassica nigra*, 37% of its total DNA bands as a satellite peak; at the other extreme, *B. oleracea* has no satellite at all. Seven other species have satellite amounts which fall between these two extremes (Beridze, 1975). American species of *Phaseolus* (*P. vulgaris*, *P. multiflorus* and *P. lunatus*) have about 30% satellite DNA, Asian species (*P. angularis*, *P. calcaratus*) have less, about 10-15% satellite, whilst *P. aureus* (also an Asian species) does not show any satellite in a CsCl density gradient (Beridze, 1972). This led to the hypothesis that if these nuclear satellites arose by saltatory

replication then *P. aureus* is the most primitive species and Asian species, as a whole, are more ancient than their American relatives.

Variation of satellite DNA content is also found among closely related genera of the same family. Ingle et al. (1973, 1975) studying 59 species of dicots and 26 species of monocots found that species having more than 3 pg (10^{-12} g) of DNA did not show any satellite band in CsCl gradients and suggested that this might be a general phenomenon. At this stage, however, it is impossible to rule out chance coincidence since the study is no way exhaustive.

5. *Localization of Satellite in the Chromosome*

Highly repeated sequences or satellites are arranged in long tandem arrays in DNA (Bond et al., 1967; Gall and Atherton, 1974; Goldring et al., 1975; Brutlag et al., 1977). In *Drosophila melanogaster* there are ten regions of 1.705 (g/cm³ density) satellite sequences in the entire haploid genome; each region extending about 750 kilobases (Brutlag et al., 1977). *In situ* hybridization studies in this species reveal that the highly repeated sequences are mostly localised in the constitutive heterochromatin associated with the centromeres. The pattern of distribution of satellite DNA sequences within the heterochromatic blocks is usually unique for each chromosome (Prescott et al., 1973; Jones et al., 1974; Appels et al., 1978). Satellite DNA hybridizes specifically to constitutive heterochromatin but not all such regions necessarily contain satellite DNA. They do, however, appear to contain highly repeated DNA.

Satellite sequences in *Scilla sibiricus* are distributed in the heterochromatic regions of the nuclei and on chromosomes known to be heterochromatic (Timmis et al., 1975). In *Tropaeolum majus* satellite DNA is localised at the nucleolus-associated heterochromatin (Deumling and Nagl, 1978). Highly repeated (C_0t 0.02) DNA sequences of rye are localized mainly within c-bands near the distal ends of most chromosome

arms (Appels et al., 1978).

6. Functions of Satellite DNA

At present the function of highly repeated DNA is unclear, though many suggestions have been made. The fact that a short monotonous stretch of nucleotides is repeated thousands of millions of times, that nonsense codons are included in many cases, and that the preferred location of the highly repeated sequences is in the regions of chromosomes that are heterochromatic, all suggest that such sequences are probably not transcribed. It is hard to believe that a cell will maintain a large amount of DNA without any function and yet conserve its sequences. There is, however, some evidence that parts of these sequences may be transcribed (Straus and Birnboim, 1976). The basis of this suggestion is the observation that some polypyrimidine tracts ("uninterrupted runs of pyrimidine nucleotides, averaging 750 residues in length", Birnboim and Sederoff, 1975) can react 'extensively' with total cellular RNA. Hybridization of ribosomal RNA to some satellites (tomato, Chilton, 1975; *Phaseolus coccineus*, Lima-de-Faria et al., 1975) does not necessarily mean that highly repeated DNA are transcribed, because ribosomal DNA sequences may be separated along with the satellite in a CsCl gradient and ribosomal sequences do not usually belong to the category of highly repeated DNA.

Although based on no experimental evidence, Walker (1971, 1978) suggested that such sequences perform some mechanical role of 'house-keeping' and are involved in arranging functional sequences in relation to higher order chromatin structure. It has also been suggested that the chromosomal specificity of satellite DNA sequences may play a role in homologous chromosome recognition in the cell (Brutlag and Peacock, 1975). Considering all the evidence available at present, John and

Miklos (1979) have concluded that the function of only some types of satellite is known for certain and that these play a role only in the germ line. One function of this type appears to be the regulation of recombination between homologous chromosomes at meiosis.

7. *Role of satellite DNA in Differentiation and Evolution*

A general, but not absolute, relationship exists between genome size and the degree of structural complexity (Kohne, 1970; Price, 1976). In many plants an increase in DNA content is attributed, largely if not exclusively, to an increase in the amount of repetitive DNA (Flavell et al., 1974), even among species within a genus (*Gossypium*, Wilson et al., 1976; *Lathyrus*, Narayan and Rees, 1977). The situation in seven *Allium* species is different. Here Ranjekar et al. (1978b) found no significant change in the amount of repetitive sequences present in different species, although there was a three-fold variation in their DNA content.

Variation in the amount of satellite DNA among different tissues of the same species has been observed in melon (Pearson et al., 1974) and *Phaseolus* (Lima-de-Faria et al., 1975). Meristematic tissues (seed, root tips) of melon were shown to contain a higher percentage of satellite than mature, differentiated tissues (fruit, cotyledon, leaf). In *Phaseolus*, the cells of the integument contain the highest amount (34.7%) of satellite (density 1.700 g/cm³) and roots contain the lowest (28.2%). Suspensor cells have 32.9% of this satellite and an additional satellite, density 1.696 g/cm³. It was argued that r-RNA genes are underreplicated in suspensor cells and that this was partially cancelled out by the appearance of 1.696 satellite. Differential amounts of the same satellite in different tissues may be the result of differential amplification (or under replication) of the satellites. In *Cymbidium* the A + T-rich

satellite was found to be amplified only in a certain population of nuclei (Nagl, 1977). It is not clear though whether the difference in the amount of satellite DNA in different tissues is the cause or the consequence of differentiation.

Enhancement of satellite DNA synthesis after hormone treatment has been reported for cucumber (Kadouri et al. 1975). Heterochromatin is believed to undergo differential replication in relation to cellular differentiation; it is underreplicated in pea (van Oostveldt and van Parijs, 1976) and *Tropaeolum majus* (Nagl et al., 1976) and over-replicated in *Cymbidium* (Nagl and Rucker, 1976). Parenti et al. (1973) observed a transient satellite which is present only between 48 and 72 hours after culture of pith tissue of *Nicotiana glauca* and argued that this may be the cause of the phase change which, in turn, leads to callus formation. There is a report of differential satellite synthesis in the tumorous cells of *Crepis*, (Sacristan and Dobrigkeit, 1973) but the evidence is not convincing.

Conservation of sequences in related species, differential amounts in different species, and the fact that a significant portion of all eukaryotic genomes consists of highly repeated DNA, all suggest that these sequences are important in evolution and speciation. According to Nagl (1978), this "non-coding, highly reiterated, simple sequence DNA, is involved in ontogenetic differentiation and phylogenetic diversification". It was concluded that heterochromatin (satellite DNA) controls both the gross morphology of karyotypes and chromosomes and their evolution. For this reason, the genetically inert, highly repetitive DNA has been called 'chromosome engineering DNA' (Nagl, 1978).

7. Satellite DNA and Contamination by micro-organisms in Plants

Micro-organisms, especially bacteria, exist in some or all parts of plants (Lange, 1966; Lewis et al. 1977; Dunleavy and Urs,

1973). Unless precautions are taken to eliminate or reduce their number, the bacterial DNA may band as a satellite in the isopycnic centrifugation of total DNA and so lead to erroneous conclusions. It is already known that novel satellites originating under stress conditions (Quetier et al., 1968), or after wounding, are contributed by bacterial DNA (Pearson and Ingle, 1972; Broekaert et al., 1975). Rapid labelling of a portion of plant DNA in response to hormonal treatment has also been shown to arise from bacterial contamination (Sarrouy-Ballat et al., 1973).

Bendich (1972) has suggested a method for eliminating, or at least reducing, the bacterial contamination problem in plant DNA preparations. Unfortunately this method is of little use if one wants to know the extent of bacterial contamination before extracting DNA from plant material. Applying the seed sterilization technique of Halsall and Forrester (1977) and Halsall (1978), a reduction of barley associated bacteria can be achieved to a level that does not confound the recognition of satellite DNA (see Chapter II).

Scope of Thesis

The genus *Hordeum* is a monocot belonging to the family Gramineae. There are two schools of thought regarding the origin of cultivated barley (*H. vulgare*). One school believes that present day cultivated barley originated from a six-rowed ancestor, *H. agriocrithon* (Bakhteyev, 1964, 1970); the other argues that it originated from the two-rowed *H. spontaneum* (Zohary, 1959, 1964, 1970; Staudt, 1961; Helback, 1965; Harlan, 1976). Arguments relating to ancestry are based mainly on distribution, morphology, adaptation, interspecific crosses and archaeology. At the time when *H. spontaneum* was accepted as the putative ancestor of cultivated

barley, Csizmarik (1976) put forward the hypothesis, based on statistical analysis, that a six-rowed barley was the progenitor. He concluded that "the evidence on which this result is based is archaeological, as well as the historical time-framework into which plant diffusion must be fitted". From the weight of evidence, it now seems that *H. spontaneum* is indeed the ancestor of present-day cultivated barley, and that most probably *H. agriocrithon* is a hybrid between *H. spontaneum* and cultivated barley.

The genus *Hordeum* contains nearly thirty species, only one of which (*H. vulgare*) is used as a grain crop mainly for animal feed, brewing malts and human consumption. The genus contains species of different natural ploidy levels (2x to 6x). In some cases genome analysis has provided information on the possible donor(s) of different genomes in the tetraploid and hexaploid forms (Rajhathy et al., 1964; Stark and Tai, 1974; Wagennar, 1960).

During the period that has elapsed since this research began in late 1975 there has been a considerable growth of information on the molecular biology of plant genomes. Prior to 1975 the DNA of *H. vulgare* was studied in the context of its similarity or divergence in base sequences to those of wheat, rye and oat (Bendich and McCarthy, 1970a; Smith and Flavell, 1974). In parallel with this research, two studies on the repetitive DNA of *H. vulgare* were reported (Ranjekar et al., 1976, 1978a). Nothing is known about the DNA of other species however.

This thesis is directed towards an understanding of molecular changes associated with speciation in *Hordeum*. The DNA of three closely related diploid species (*H. vulgare*, *H. spontaneum* and *H. agriocrithon*) are compared both between themselves and with DNAs from a phylogenetically distinct group of species (*H. californicum*

(2x), *H. jubatum* (4x) and *H. arizonicum* (6x) known to be related to one another in having one genome in common and forming a natural polyploid series. The DNA of *H. vulgare* and *H. arizonicum* have been chosen for detailed analysis as representative of these groups. The results of these comparisons form the basis of this thesis and are summarised in the five Chapters that follow this Introduction. In sequence they are (i) the influence and minimisation of the effect of bacterial components on the analysis of DNA; (ii) buoyant density and thermal dissociation/reassociation studies; (iii) physical characterisation and chromosomal localization of cryptic satellites from *H. vulgare* and *H. arizonicum*; (iv) *in situ* hybridization studies of H3 (*H. vulgare*) and H1 (*H. arizonicum*) satellites with chromosomes of other *Hordeum* species and (v) general discussion.

CHAPTER II

THE INFLUENCE AND MINIMISATION OF THE EFFECT OF BACTERIAL COMPONENTS ON ON THE ANALYSIS OF DNA

INTRODUCTION

Previous studies to ascertain the relationship between different species of *Hordeum* have been restricted to the analysis of meiotic chromosome pairing. With the advent of new techniques in molecular biology, one can achieve more meaningful estimates of the extent of relatedness or divergence between species by subjecting their DNA(s) to physicochemical analysis. The validity of such comparative analyses depend primarily on the degree of purity of the respective plant DNAs. Recent studies have revealed both bacteria and viruses associated with *Panicum* (Lewis and Crotty, 1977) and soybean (Dunleavy and Urs, 1973). Others (Bendich, 1972; Ingle et al., 1973; Delseny, 1975; Sarrouy-Ballat et al., 1973; Quetier et al., 1968) warn that DNA of contaminating bacteria may lead to erroneous conclusions. For example, DNA satellites initially thought to be of plant origin were later shown to arise from contaminant DNA (Delseny, 1975; Bendich, 1972; Pearson and Ingle, 1972; Broekaert and Parijs, 1975).

Based on the recovery of DNAs of intermediate densities when plant seedlings were treated with pure DNA isolated from *Micrococcus lysodeikticus*, Ledoux and Huart, 1969 and 1975, claimed to have demonstrated the uptake, integration and replication of exogenously supplied bacterial DNA by plant material, including barley. Kleinhofs and his co-workers (Kleinhofs et al., 1975; Kleinhofs, 1975) were, however, able to demonstrate the disappearance of such intermediate DNA components following rigorous sterilisation of recipient seeds and suggested that these DNA components may have arisen from contaminating bacteria.

This Chapter describes: (1) the isolation of barley associated bacteria, (2) the nature of their DNAs, and (3) methods of minimising bacterial DNAs in DNA preparations of barley. In the light of the present findings, claims for DNA uptake, integration and replication of foreign DNA by plant material are discussed. A preliminary report has appeared elsewhere (Chakrabarti et al., 1978a).

MATERIALS AND METHODS

Seeds of *Hordeum vulgare* c.v. clipper were obtained from Dr. R. Oram, Division of Plant Industry, CSIRO, Canberra.

Treatment of Seeds

For microbial sterilisation tests, seeds were rinsed with one of the following: (a) sterile distilled water (control); (b) 5% NaOCl solution for 15 min. followed by two rinses with sterile distilled water; (c) a solution containing per litre, HgCl_2 , 0.5 g; conc. HCl, 1.25 ml; NaCl 6.5 g and ethanol, 250 ml, for 5 min. followed by soaking in 50% ethanol for 10 min. and two rinses in sterile distilled water (Williams et al., 1974); (d) 50% H_2SO_4 (v/v) for 1 hr and 5% calcium hypochlorite for 30 min. (Kleinhofs et al., 1975); (e) 1% AgNO_3 for 2 min. followed by drying on sterile filter papers (several changes) (Halsall and Forrester, 1977; Halsall, 1978).

Germination of Seeds

Seeds (100 seeds per Petri dish, diam. 15 cm) were germinated on: (i) sterile wet filter paper; (ii) Luria agar; (NaCl, 5 g; yeast extract, 5 g; Tryptone, 10 g; Difco Bacto Agar, 10 g/l); (iii) Difco Bacto Agar, 10 g/l; (iv) agar plates containing Difco Bacto Agar, 1% w/v and 1 mg/ml Benzylpenicillin (Commonwealth Serum Laboratories,

Melbourne, Australia).

Petri dishes 'inoculated' with seeds were sealed with Nescofilm and incubated at 22 C in the dark for 7 to 10 days. By this time germination has advanced to the point that roots and shoots were several cms long with the roots in intimate contact with the surface of the medium.

Isolation of micro-organisms and their Growth

Fresh tissues were ground in a mortar with pestle. Extracts of root and shoot tissues were placed on Luria agar, potato dextrose agar (Booth, 1971a) and Czapek-Dox (Booth, 1971b) agar and incubated at 30 C under both aerobic and anaerobic conditions. The different media all grew similar numbers of colonies aerobically and each distinct type of colony contained bacteria rather than other micro-organisms. Although the different media altered the appearance of colonies, the same number of types were recognised on each. Growth was standardised to Luria plates, aerobically at 30 C. Since extracts made in Luria broth or in DNA extraction buffer (0.15 M NaCl, 0.1 M EDTA; pH 8.0) gave the same number of bacterial colonies, all extracts were made in the extraction buffer.

DNA Extraction

Roots and shoots of 7 to 10 day-old seedlings were harvested separately in a laminar flow hood. Fresh tissue was frozen in liquid N₂ and ground in a mortar with pestle. The rest of the procedure was essentially similar to that of Marmur (Marmur, 1963). All steps were carried out at 4 C under sterile conditions. Bacterial DNA was extracted following the procedure of Marmur (Marmur, 1963).

DNA was purified by isopycnic centrifugation gradients using a Beckman L2-65B preparative ultracentrifuge. DNA solutions (mixed with CsCl) of initial density 1.705 g/cm³ were run in a 50 Ti rotor at

44000 rpm at 20 C for at least 48 hr. Sometimes repeated preparative steps were necessary to obtain 260/280 and 260/230 absorbance ratios of greater than 1.9. Fractions were collected by puncturing the bottom of the tube with a 17 gauge needle. Absorbancy was measured in a Gilford 2400 Spectrophotometer.

RESULTS

Control plates inoculated with seeds rinsed with sterile distilled water, yielded confluent bacterial growth around roots and shoots within 2 days of incubation at 30 C (Table I). Extracts contained between 10^8 and 10^{10} bacteria/gram of tissue (Table II).

Seeds treated with NaOCl were not sterilised (Table I) and extracts of root tissues showed a contamination level of nearly 10^9 bacteria/gram of tissue (Table I). Four types of bacteria (based on the visual appearance of colonies) were isolated by plating the extracts as dilution series on Luria agar. These isolates were termed B₁, B₂, B₃ and B₄ and, as pure cultures, were used as sources of bacterial DNA. The same types of bacteria were obtained from shoot extracts but counts were about 10-fold lower (Table I).

Treatments with either HgCl₂ or H₂SO₄ followed by Ca (OCl)₂ were moderately or extremely effective in sterilisation but proved detrimental to germination (Table II).

Silver nitrate treated seeds germinated well and no microbial growth was observed on culture medium even after 7 days (Table II). Exposure to Luria agar is a very sensitive test for sterility, since the bacteria referred to above when plated on this medium grow to observable colonies overnight. Even so, extracts of seemingly sterile root tissue, taken from Luria plates used for seed germination, yielded between 10^5 and 10^6 bacterial colonies/gram of tissue (Table I).

However, only two types of bacteria (B_1 and B_2 usually in similar proportions) were found in extracts resulting from seeds treated with $AgNO_3$. Thus, $AgNO_3$ is by far the most effective sterilising agent for barley seeds, reducing the numbers of bacteria by nearly 4-orders of magnitude and halving the number of types of bacteria. Moreover, this was achieved without a noticeable effect on seed germination.

TABLE I

Extent and types of bacteria in the extracts of barley seedlings
following different treatment and planting on Luria agar

Treatment	Number of bacterial colonies/g of tissue		
	Root	Shoot	Types of bacteria (minimum)
Control	1.05×10^9	2×10^8	B_1, B_2, B_3, B_4
Sodium hypochlorite	6.67×10^8	7.00×10^7	B_1, B_2, B_3, B_4
Silver nitrate	4.06×10^5	3.64×10^4	B_1, B_2

Penicillin was used in an attempt to achieve complete sterilisation. Inhibitory concentrations of penicillin for B_1 and B_2 were found by direct plating to be 10 $\mu\text{g/ml}$ and 1000 $\mu\text{g/ml}$ of Luria medium. Silver nitrate treated seeds germinated on Luria agar containing penicillin (1000 $\mu\text{g/ml}$) showed no microbial growth after 7 days but root tissue extracts when plated on Luria agar still gave similar bacterial counts.

Under all conditions of seed treatment and germination the level of contamination of shoot tissues was about one order of magnitude less than for root tissues (Table I). Shoot DNA, irrespective of the

TABLE II

Effect of different seed treatments and method of germination
on visible 'contamination'

Germination on	Method of seed treatment				
	Sterile water	NaOCl	HgCl ₂	AgNO ₃	H ₂ SO ₄ and CaOCl
Wet filter paper	++++	++++	not done	-	not done
Luria agar	++++++	++++++	++ [*]	-	not done
Plain agar	++++	++++	- [*]	-	- [†]
Plain agar + 1000 µg/ml penicillin	+++	+++	not done	-	not done

+ degree of bacterial growth

- no visible bacterial growth

* germination 20-25%

† poorly developed shoot and root system

efficiency of sterilisation of seed bacteria, always showed a single sharp peak at a density of $1.700 \pm 0.001 \text{ g/cm}^3$ in a neutral CsCl gradient (Fig. 1). This is presumably because of the lower level of bacteria and what appeared to be a greater ease of grinding shoots and extracting their DNA, thereby enhancing the ratio of plant to bacterial DNA.

The situation was very different for roots. Following inadequate seed treatment, DNA from roots displayed an additional multi-component band in a neutral CsCl gradient with three 'peaks' at buoyant densities 1.714, 1.719 and 1.724 g/cm^3 which, together, accounted for up to 30% of the

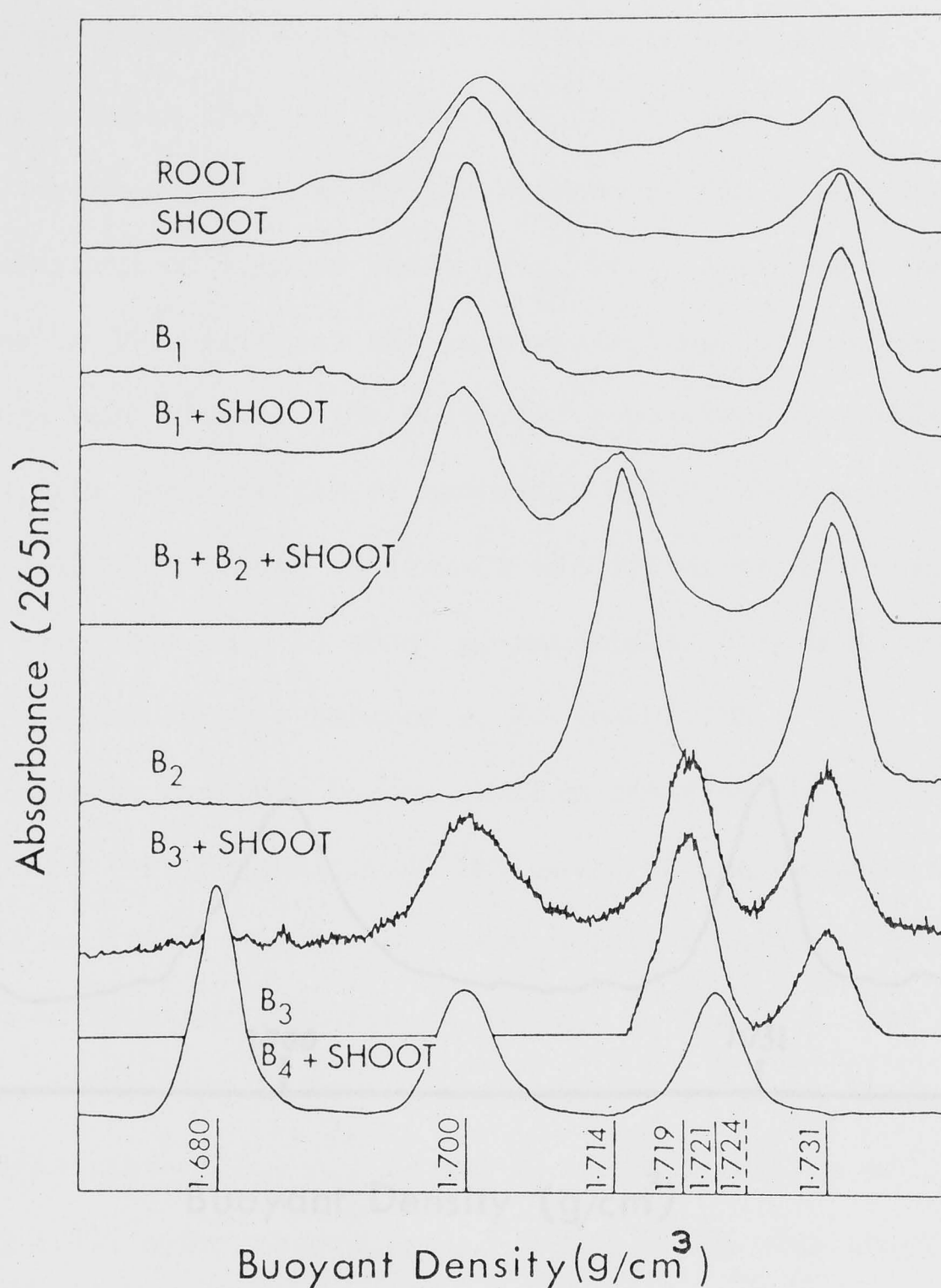


Fig. 1. Buoyant density profiles in neutral CsCl of barley and bacterial (isolated from barley roots) DNA's. In all cases marker DNA is present at 1 μ g, usually *M. luteus* DNA ($\rho = 1.731$) but otherwise poly (dA-dT) ($\rho = 1.680$). The root and shoot DNA profiles are from unsterilised seeds germinated on wet sterile filter paper and, for root, should be compared with the profile of Fig. 2, representing material from AgNO_3 sterilised seeds. Bacterial DNA (B_1 , 2, 3 and 4) was used at 1-2 μ g. The tracings are as obtained and variations in area of standards and of noise are due to machine settings.

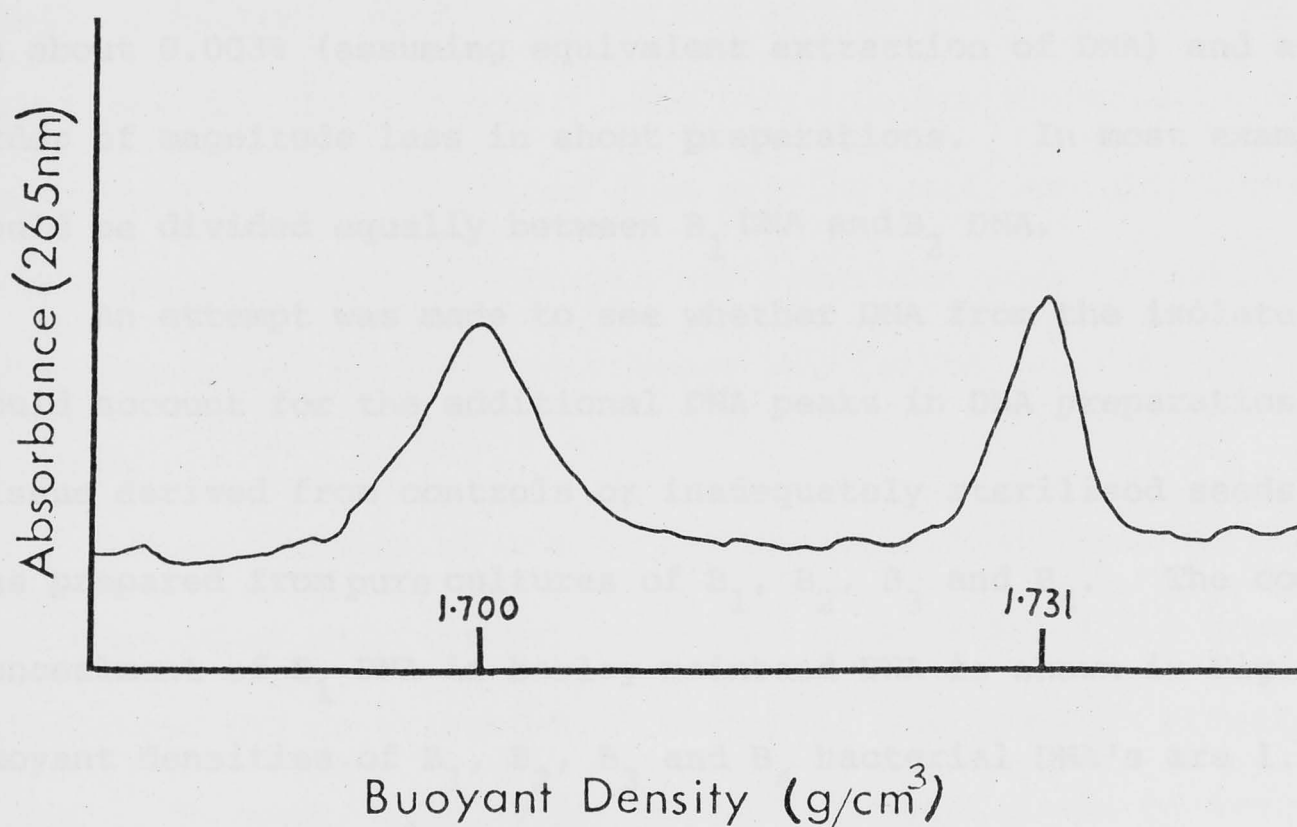


Fig. 2. Buoyant density profiles in neutral CsCl of barley root DNA when seeds were sterilized using AgNO_3 . Marker: *M. luteus* DNA ($1.0 \mu\text{g}$, 1.731 g/cm^3), plus barley root DNA ($1.0 \mu\text{g}$, buoyant density calculated as 1.700 g/cm^3).

total DNA (Fig. 1). When the DNA preparations were subjected to complete digestion by DNAase no band was detectable in analytical CsCl gradients, thus demonstrating that all peaks were due to DNA. The effectiveness of AgNO_3 treatment is shown by the absence of DNA other than main band in root extracts of treated seeds (Fig. 2). Assuming a maximum contamination of 30% bacterial DNA in preparations from untreated seeds then, since AgNO_3 reduces the bacterial population by about 4-orders of magnitude, the contribution of bacterial DNA in root preparations would be about 0.003% (assuming equivalent extraction of DNA) and at least an order of magnitude less in shoot preparations. In most examples this would be divided equally between B_1 DNA and B_2 DNA.

An attempt was made to see whether DNA from the isolated bacteria would account for the additional DNA peaks in DNA preparations of root tissue derived from controls or inadequately sterilised seeds. DNA was prepared from pure cultures of B_1 , B_2 , B_3 and B_4 . The complete concealment of B_1 DNA in barley mainband DNA is shown in Fig. 1. The buoyant densities of B_1 , B_2 , B_3 and B_4 bacterial DNA's are 1.700, 1.714, 1.719 and 1.721 g/cm^3 respectively. When mixed with shoot DNA (free of significant additional peaks) the peaks contributed by B_2 and B_3 DNA coincide with two of the additional peaks. However, DNA from B_4 could not be a significant contributor to the 1.724 g/cm^3 peak present in the root which remains unaccounted for.

DISCUSSION

At least four types of bacteria (B_1 to B_4) are associated regularly with *Hordeum vulgare* tissues and similar experiments revealed that at least three of the four types of bacteria reported here were common to five other *Hordeum* species used in this study (Chakrabarti et al., 1978b). Others have reported that micro-organisms are associated with a variety of plant tissues (Lewis and Crotty, 1977; Dunleavy and

Urs, 1973; Lange, 1966; Mundt and Hinkle, 1976). It is not known whether these micro-organisms should always be regarded as contaminants or whether associations may have a deeper biological significance.

Silver nitrate treatment is effective in reducing the level of bacterial contamination without altering the germination of treated seeds, so that the contribution of bacterial DNA is negligible. Nevertheless, two types of bacteria (B_1 and B_2) continue to be present in the extracts of tissues, even though such treatment does not allow any growth of bacteria outside roots and shoots under the most favourable conditions for the bacteria. The bacteria are therefore either within the tissues or else inhibited from growing until the plant material is ground and extracted. Bacteria are sometimes seen in root squashes but transmission electron microscopy has failed, so far, to locate the bacteria.

The sensitivity of the barley associated bacteria to penicillin (and some other antibiotics) suggested possibilities for completely eliminating the contribution of bacterial DNA from DNA preparations of barley. However, germination of $AgNO_3$ treated seeds on Luria agar plates containing penicillin did not result in reduced levels of bacterial ($B_1 + B_2$) populations in barley extracts, although these types are penicillin-sensitive. Thus the bacteria are protected from the antibiotic either by an accessibility barrier, deactivation of the drug or because the bacteria, if in contact with penicillin, are not growing.

The inhibitory effect of $HgCl_2$ or H_2SO_4 plus $Ca(OCl)_2$ treatments on seed germination may be direct but, alternatively, it cannot be ruled out that complete sterilisation of bacteria results in poor germination.

The correlation between high levels of bacterial counts from root tissue extracts, the presence of additional buoyant density peaks in the corresponding root DNA, and the disappearance of these peaks from DNA

preparations when the degree of contamination is reduced by effective treatments, suggests that the additional peaks are due to the DNA from the associated bacteria in the root tissues. The fact that the buoyant densities of two of these peaks (1.714 and 1.719 g/cm^3) correspond to those of the DNAs from the B_2 and B_3 bacteria, strongly supports this conclusion. It should be realised that no attempt was made to isolate all the different kinds of bacteria present. The four chosen were isolated merely because they were of obviously different colony appearance. Many different bacteria of similar colony appearance may have been present. Indeed the presence of a 1.724 g/cm^3 peak in DNA from non-sterilised or inadequately sterilised material indicates this, since it cannot be accounted for entirely by B_4 DNA (1.721 g/cm^3) which must be only a minor contributor. No attempt was made to isolate the organism(s) responsible for the 1.724 g/cm^3 DNA nor to identify the isolated bacteria. The interest was in the principle that bacterial DNA might contribute to the total DNA rather than in identifying the bacteria themselves and the concern was to eliminate or minimise this unwanted DNA.

It is of particular interest that the buoyant density of DNA from bacterium B_1 (1.700 g/cm^3) coincides with that of the barley DNA so that the two cannot be distinguished from one another. This emphasises that DNA forming a single sharp peak in a neutral CsCl gradient does not necessarily mean that it is of one kind.

Ingle *et al.* (1973) estimated that at contamination levels of 10^4 to 2×10^6 bacteria/gram of plant tissue, the contribution of bacterial DNA could be 0.0005% to 0.1% of the total DNA. In the present experiments 4.6×10^5 viable bacteria/gram of root tissue from the AgNO_3 treated seeds probably contribute about 0.003% of the total DNA. This would be divided about equally between DNA from B_1 and B_2 . The buoyant

density of B_2 DNA (1.714 g/cm^3) allows it to be separated from main band barley DNA in a preparative CsCl gradient. Contamination of barley root DNA by bacterial DNA can therefore be reduced to about 0.0015% due to the remaining B_1 DNA.

If shoot DNA is used, even from non-sterilised or inadequately sterilised seeds, then the contribution of bacterial DNA is inherently less. No matter whether seeds were germinated on Luria plates or under clean glasshouse conditions, the presence of bacterial DNA was never a practical problem with shoot DNA preparations.

The experiments show that once bacterial DNA is reduced to a negligible amount no significant barley satellite DNA can be detected by CsCl gradient analysis. This is not to say that cryptic satellites (hidden in mainband DNA) do not exist, in fact they do (Ranjekar *et al.*, 1976; Deumling *et al.*, 1976) and Chapters III and IV of this thesis describes the pattern of cryptic satellites present in six barley species and discuss how these may be used to study genetic relatedness.

The presence of DNA of intermediate buoyant densities (1.712 , 1.711 to 1.718 , and 1.723 to 1.727 g/cm^3) between that of the recipient DNA (barley, 1.702 g/cm^3) and of the donor bacterial DNA (*Micrococcus lysodeikticus* 1.731 g/cm^3) was taken by Ledoux and Huart (1969, 1975) as evidence for uptake, integration and replication of foreign DNA in plants. Others, reinvestigating the problem, attributed these intermediate peaks to contaminant micro-organisms, since they were not found when seeds were treated rigorously to avoid contamination (Kleinhofs *et al.*, 1975; Kleinhofs, 1975). The buoyant densities of the various bacterial DNA's found herein correspond well with those earlier attributed to contaminants. Since different *Hordeum vulgare* genotypes were used in the separate investigations in different parts of the world, it seems likely that the same or similar bacteria are commonly associated with barley of different geographic regions. Of particular interest in terms of the present results is the fact that Ledoux and Huart (1975) found that DNA

from shoots contained little, if any, 'integrated' bacterial DNA, their claim resting on seemingly large amounts in root DNA. Clearly the validity of any claim for hybrid bacterial-plant DNA must depend in part on an investigation of the level and nature of bacterial or bacterial DNA contamination of root extracts and the rigorous elimination of this as a contributing factor. Due to the difficulties encountered in eliminating bacteria from barley seeds using traditional methods, the seeming universality of the contamination problem, and the present determination of buoyant densities of the bacterial DNA's concerned, I concur with the suggestion (Kleinhofs, et al., 1975) that the claim for hybrid bacterial-plant DNA is based on inadequate evidence. It is, however, clear that the AgNO_3 sterilisation technique could be used as a starting point for those who might wish to retest the direct transformation of barley by germinating seeds in the presence of bacterial DNA.

CHAPTER III

BUOYANT DENSITY AND THERMAL DISSOCIATION/REASSOCIATION STUDIES OF BARLEY

(HORDEUM) DNA

Genome relationship studies between *Hordeum* species have been restricted to meiotic chromosome pairing in interspecific hybrids (Rajhathy et al., 1963; Starks and Tai, 1974). Following the observation of non-homologous chromosomal associations in monohaploids (Sadasivaiah and Kasha, 1971, 1973) there are serious doubts about the validity of such analyses in experimental hybrids. As pointed out in Chapter I, an alternative and direct approach for phylogenetic studies is the use of biochemical and cytochemical techniques.

To assess the phylogenetic relationships between *Hordeum* species, a set of three closely related diploid species (*H. vulgare*, *H. spontaneum* and *H. agriocrithon*) and another set of three species with one genome in common and forming a polyploid series (*H. californicum* (2x), *H. jubatum* (4x) and *H. arizonicum* (6x)) were chosen. This Chapter describes the physical characteristics and satellite patterns in $\text{Ag}^+ - \text{Cs}_2\text{SO}_4$ gradients of DNA from the six species and reassociation behaviour of DNAs from two species (*H. vulgare* and *H. arizonicum*). The reason for selecting these two species is that they are both terminal products of a sequence of evolutionary changes within two natural groups of species. In parallel with the present work, two cryptic satellites were reported in *H. vulgare* DNA (Ranjekar et al., Deumling, et al., 1976) and reassociation of *H. vulgare* DNA has been described by Ranjekar, et al. (1976). These observations are discussed in the light of present findings.

MATERIALS AND METHODS

Plants

Species, ploidy and source of seeds, are listed in Table 1. Plants were grown under glasshouse conditions at 22 ± 2 C at optimal nutritional levels. Shoots, rather than roots, were the source of DNA because as shown in Chapter II, they contain less bacteria and were easier to extract. In all cases these shoots were obtained from plants grown under uniform conditions in a controlled growth chamber.

TABLE I

Species, ploidy and source of seed

Species	Ploidy $x = 7$	Source
<i>H. vulgare</i>	2x	Dr. R. Oram, CSIRO, Division of Plant Industry, Canberra
<i>H. agriocrithon</i>	2x	Dr. R. A. Finch, Plant Breeding Institute, Cambridge
<i>H. spontaneum</i>	2x	"
<i>H. californicum</i>	2x	Dr. D. R. Dewey, Utah State University, Logan, Utah, U.S.A.
<i>H. jubatum</i>	4x	"
<i>H. arizonicum</i>	6x	Dr. D. H. B. Sparrow, Waite Agricultural Research Institute, Adelaide

DNA extraction

The procedure for DNA extraction and checking the extract for bacterial contamination has been described in Chapter II. DNA was finally purified in preparative CsCl gradients (Chapter II).

Buoyant density measurements

Buoyant density of native and reassociated DNA in CsCl was determined as described in Chapter II.

Thermal dissociation

Thermal denaturation was carried out using a Zeiss spectrophotometer equipped with a Haake thermoprogrammer. DNA solutions in 0.12 M phosphate buffer (PB) (0.18 M Na^+ , pH 6.8, 1 ml) were added to cuvettes and tapered teflon stoppers were firmly placed to check evaporation. Thermal denaturation characteristics were determined, increasing the temperature from 20 C to 100 C at the rate of 1 C/min recording the change of absorbance. Relative absorbance ($A_t/A_{25 \text{ C}}$; A = Absorbance) was plotted against temperature. The temperature at which half dissociation was complete was taken as the melting temperature (T_m). *Escherichia coli* DNA (purchased from Sigma, U.S.A.) was used as a standard ($T_m = 90.5 \text{ C}$ in 0.18 M Na^+).

DNA was sheared by twice passing the DNA solution through a French pressure cell at 18000 psi. The solution was dialyzed in 0.12 M PB. Sheared DNA molecules were of uniform size (Fig. 1). The observed sedimentation coefficient ($S_{(\text{obs})}$) was determined following the procedure of Baur and Vinograd (1971) and was found to be 6.45. Since it was not the intention to obtain accurate estimates of the physical characteristic of the DNA, no corrections for $S_{(\text{obs})}$ were necessary.

Dissociation and Reassociation of DNA

Reassociation of *H. vulgare* and *H. arizonicum* DNA was carried out according to the prescription of Britten *et al.* (1974) using a Hydroxyapatite column (Britten *et al.*, 1974; Kohne and Britten, 1971). DNA in sealed tubes was denatured by heating at 100 C for 5 minutes and then incubated at 60.5 C ($T_m - 25$ C) for the times required to achieve desired C_0t values ($C_0t = \text{Molarity} \times \text{sec/litre}$). C_0t was calculated as the half product of absorbance of dissociated DNA \times hour(s) of incubation ($C_0t = A/2 \times t$ (hours)) (Kohne and Britten, 1971; Britten *et al.*, 1974). The DNA mixtures were then passed through a water jacketed column of hydroxyapatite (1.5 cm \times 4 cm) equilibrated at 60 C and 0.12 M PB, pH 6.8. First, single stranded DNA molecules were washed off the column by 0.12 M PB and then double stranded (reassociated) DNA was removed using 0.4 PB. The optical density of the fractions were measured in a Gilford 2400 spectrophotometer. The percent DNA reassociated was then plotted against C_0t on a semilogarithmic paper. *Escherichia coli* DNA (purchased from Sigma, USA) was used as standard. DNA grade hydroxyapatite was purchased from Bio-Rad, U.S.A.

Buoyant density studies in $\text{Ag}^+ - \text{Cs}_2\text{SO}_4$ gradient

The procedure was similar to that of Ranjekar (1976). The ratio of Ag^+ to DNA-phosphate (R_F value) was adjusted by adding 1 mM AgNO_3 to 55 μg DNA (absorbance 1.0/1 cm light path = 50 μg DNA/ml) in 10 mM sodium borate (pH 9.2). DNA-phosphate concentration was calculated from $A_{260\text{nm}}$ using a mean molar extinction coefficient of 6600. For $R_F = 0$, the initial density of Cs_2SO_4 was 1.45 g/cm³ and for all other R_F 's the initial density was 1.50 g/cm³. The optimum R_F for resolution of satellites from DNA for different barley species was determined by analytical centrifugation (Beckman Model E, 44000 rpm, 18 hr 20 C).

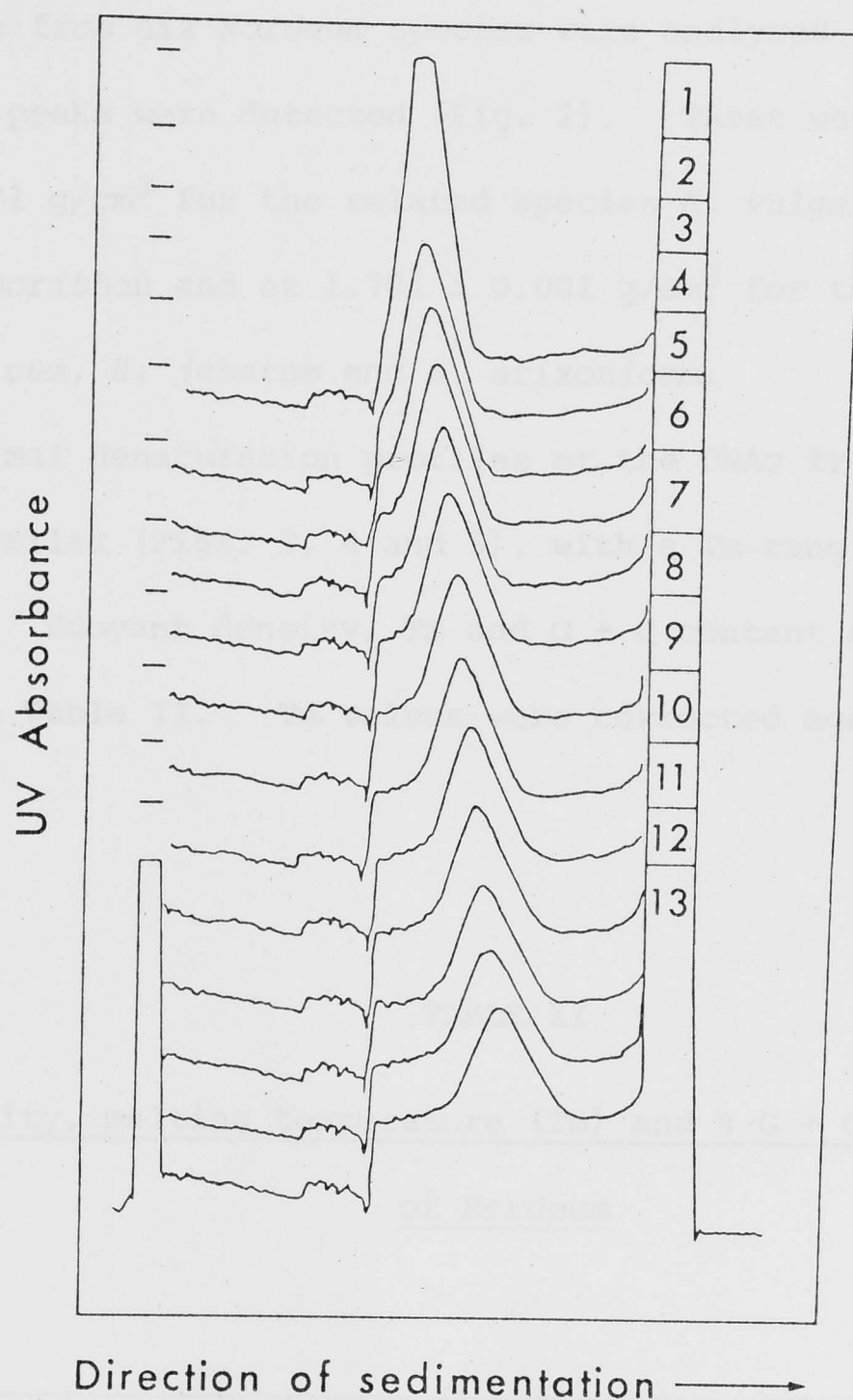


Fig. 1

Photoelectric scans of moving sheared DNA band in 1 M NaCl, 0.01 M Tris, pH 8.0 at 36000 rpm. Successive scans were taken at 5 minute intervals. Numbers on the right refer to the successive scans. Reference edges on each side were aligned.

RESULTS

Buoyant density and melting temperature

The DNAs from six *Hordeum* species were analysed in CsCl gradients. Only single peaks were detected (Fig. 2). These were observed at 1.700 ± 0.001 g/cm³ for the related species *H. vulgare*, *H. spontaneum* and *H. agriocrithon* and at 1.701 ± 0.001 g/cm³ for the polyploid series *H. californicum*, *H. jubatum* and *H. arizonicum*.

The thermal denaturation profiles of the DNAs from all six species were very similar (Figs. 3, 4 and 5), with a T_m ranging between 85.2 C and 86.2 C. Buoyant density, T_m and G + C content of all six species is listed in Table II. T_m values were corrected against T_m of *E. coli* DNA.

TABLE II

Buoyant density, melting temperature (T_m) and % G + C of different species of *Hordeum*

Species of <i>Hordeum</i>	Buoyant density g/cm ³ \pm 0.0001	TM. C	% G + C calculated from	
			density ^a	T_m ^b
<i>H. vulgare</i>	1.700	85.8	41.6	40.3
<i>H. spontaneum</i>	1.700	85.2	41.6	38.8
<i>H. agriocrithon</i>	1.700	85.8	41.6	40.3
<i>H. californicum</i>	1.701	85.4	42.3	39.3
<i>H. jubatum</i>	1.701	86.2	42.3	41.2
<i>H. arizonicum</i>	1.701	85.6	42.3	39.8

a = calculated according to Mandel et al. (1968)

b = calculated according to Mandel & Marmur (1968)

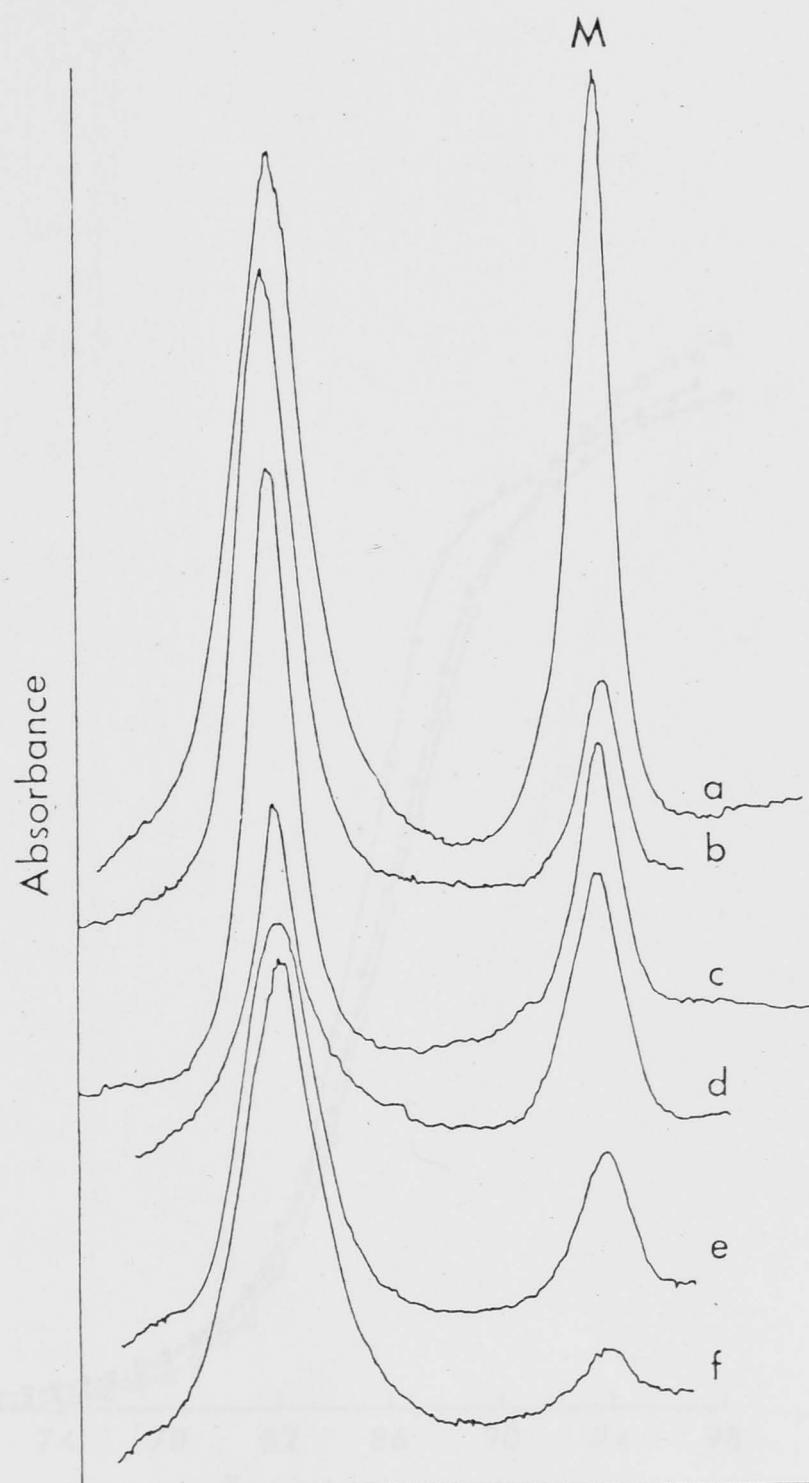


Fig. 2

Analytical ultracentrifugation of DNA of different species of barley in CsCl. (a) *Hordeum jubatum*; (b) *H. spontaneum*; (c) *H. vulgare*; (d) *H. agriocrithon*; (e) *H. arizonicum*; (f) *H. californicum*. 2-3 μg of DNA of each species was centrifuged for 18 hr at 20 C. *M. luteus* DNA ($\rho = 1.731 \text{ g/cm}^3$) was used as the marker (M).

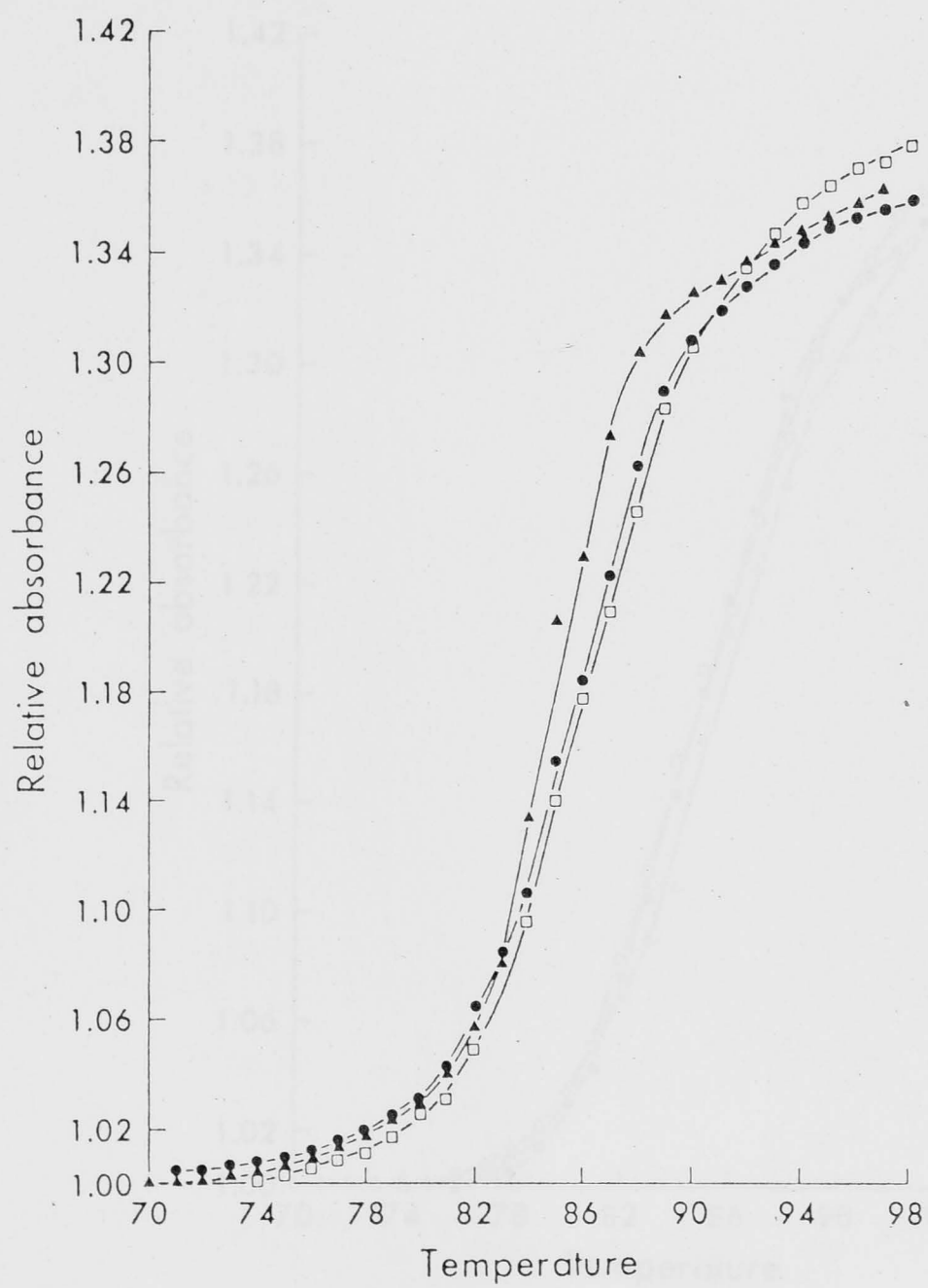


Fig. 3

Thermal denaturation profiles of DNA of related diploid species.

- (●) *H. vulgare*, $T_m = 85.8$ °C; (□) *H. agriocrithon*, $T_m = 85.8$ °C;
 (▲) *H. spontaneum*, $T_m = 85.2$ °C.

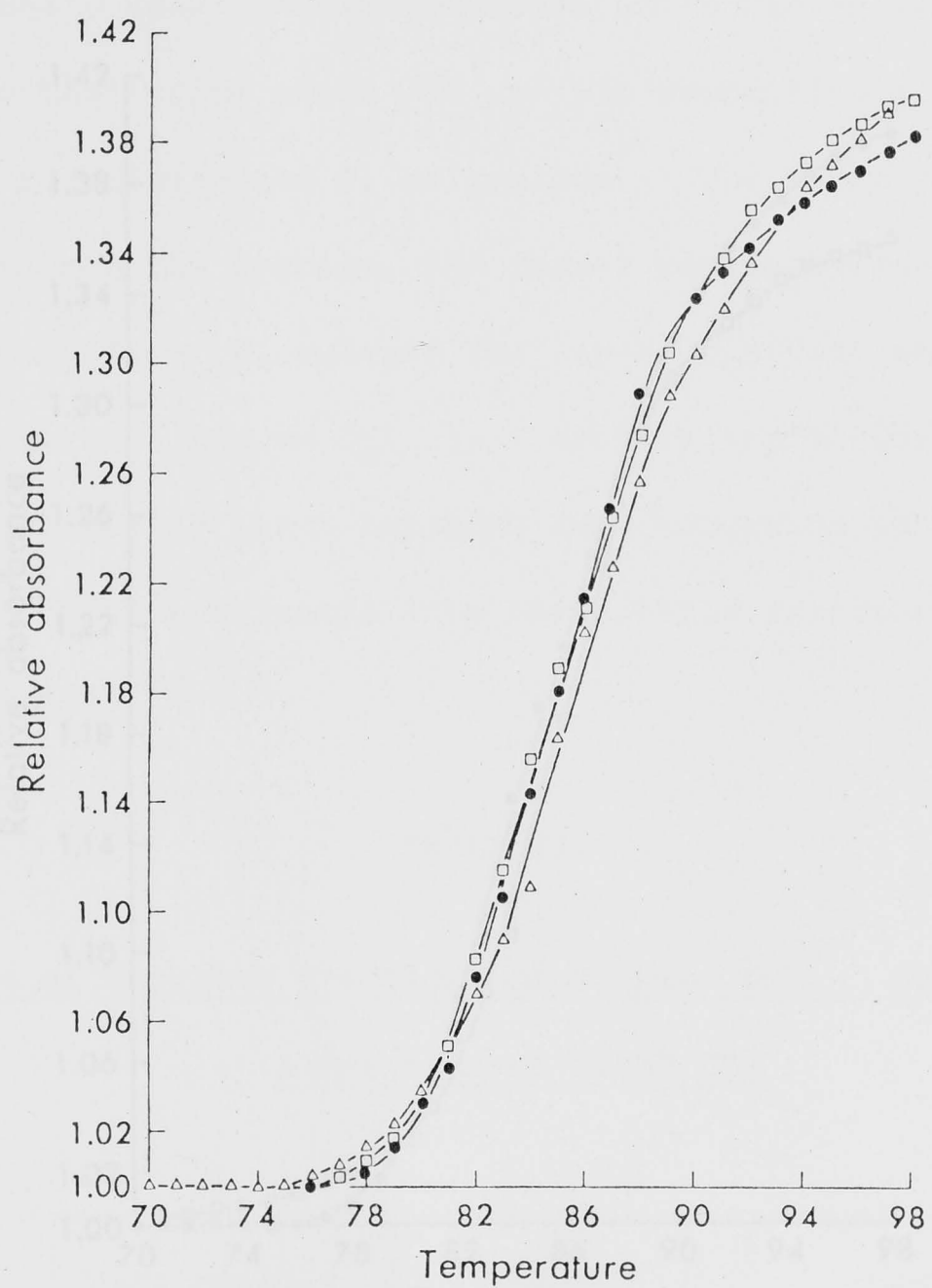


Fig. 4

Thermal denaturation profile of DNA of different species belonging to the polyploid series (●) *H. californicum* (2x), $T_m = 85.4$ C; (Δ) *H. jubatum* (4x); $T_m = 86.2$ C and (◻) *H. arizonicum* (6x); $T_m = 85.6$ C.

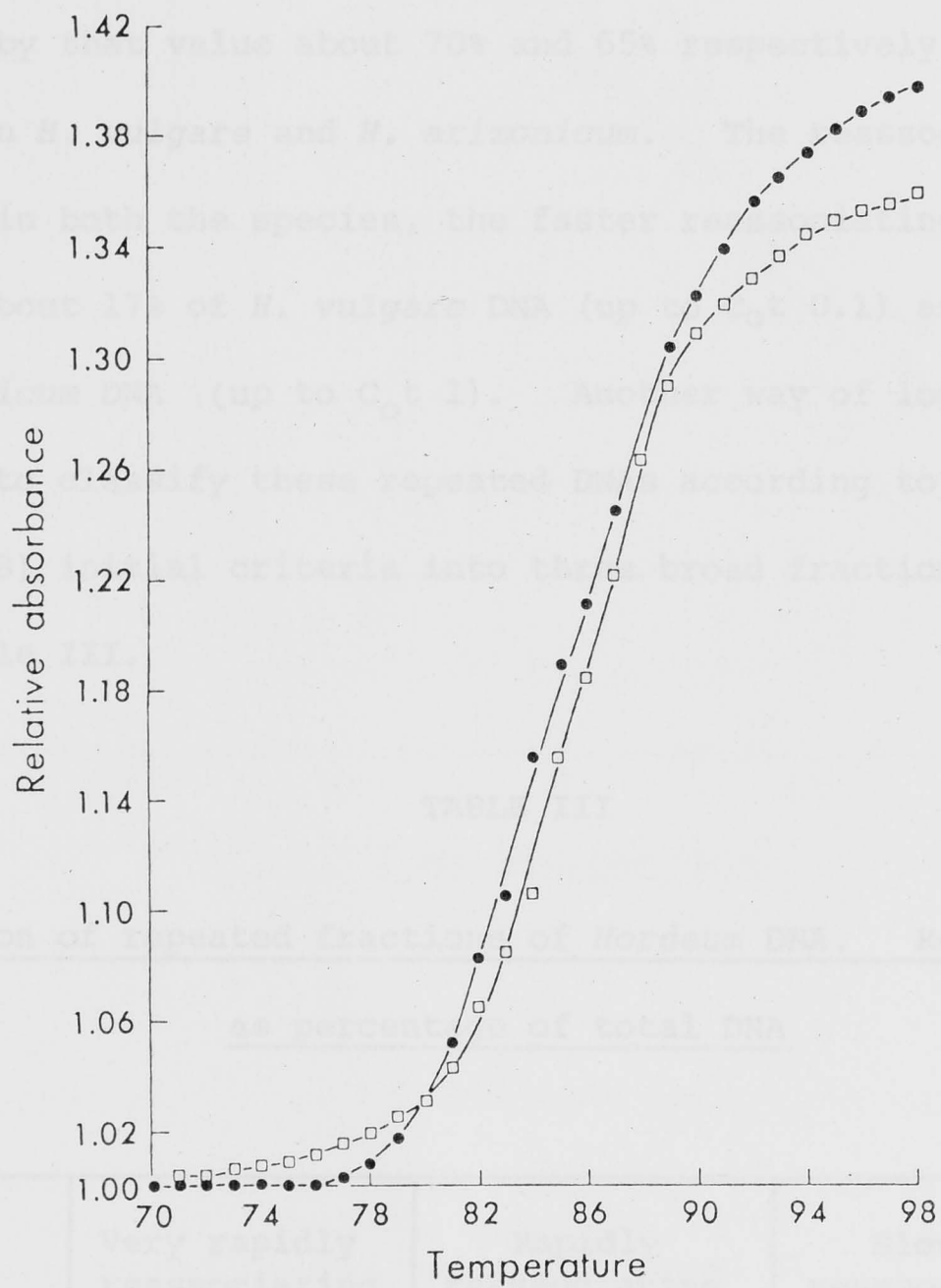


Fig. 5

Comparison of thermal denaturation profiles of *H. vulgare* and *H. arizonicum* DNA. T_m of *vulgare* DNA (□) 85.8 C and of *arizonicum* (●) DNA 85.6 C.

Reassociation and characterization of reassociated DNA

Reassociation of *H. vulgare* and *H. arizonicum* DNAs was studied over a range of C_0t values (10^{-3} – 10^2). The extent of reassociation was plotted against these C_0t values (Fig. 6). It is to be noted that it is usually the repeated sequences which reassociate up to $C_0t 10^2$ and by that value about 70% and 65% respectively of DNA has reannealed in *H. vulgare* and *H. arizonicum*. The reassociation curve is biphasic in both the species, the faster reassociating component comprising about 17% of *H. vulgare* DNA (up to $C_0t 0.1$) and about 32% of *H. arizonicum* DNA (up to $C_0t 1$). Another way of looking at Figure 6 is to classify these repeated DNAs according to Britten and Kohne's (1968) initial criteria into three broad fractions as represented in Table III.

TABLE III

Classification of repeated fractions of *Hordeum* DNA. Results expressed as percentage of total DNA

Source of DNA	Very rapidly reassociating DNA (up to $C_0t 0.01$)	Rapidly reassociating DNA ($C_0t 0.01$ – 1.0)	Slowly reassociating DNA ($C_0t 1.0$ – 100)	Total (up to $C_0t 100$)
<i>H. vulgare</i>	12.5% (8–12%) a	23.5% (34–35%) a	33% (30–34%) a	70% (76%) a
<i>H. arizonicum</i>	6%	26%	33%	65%

a = data of Ranjekar *et al.* (1976)

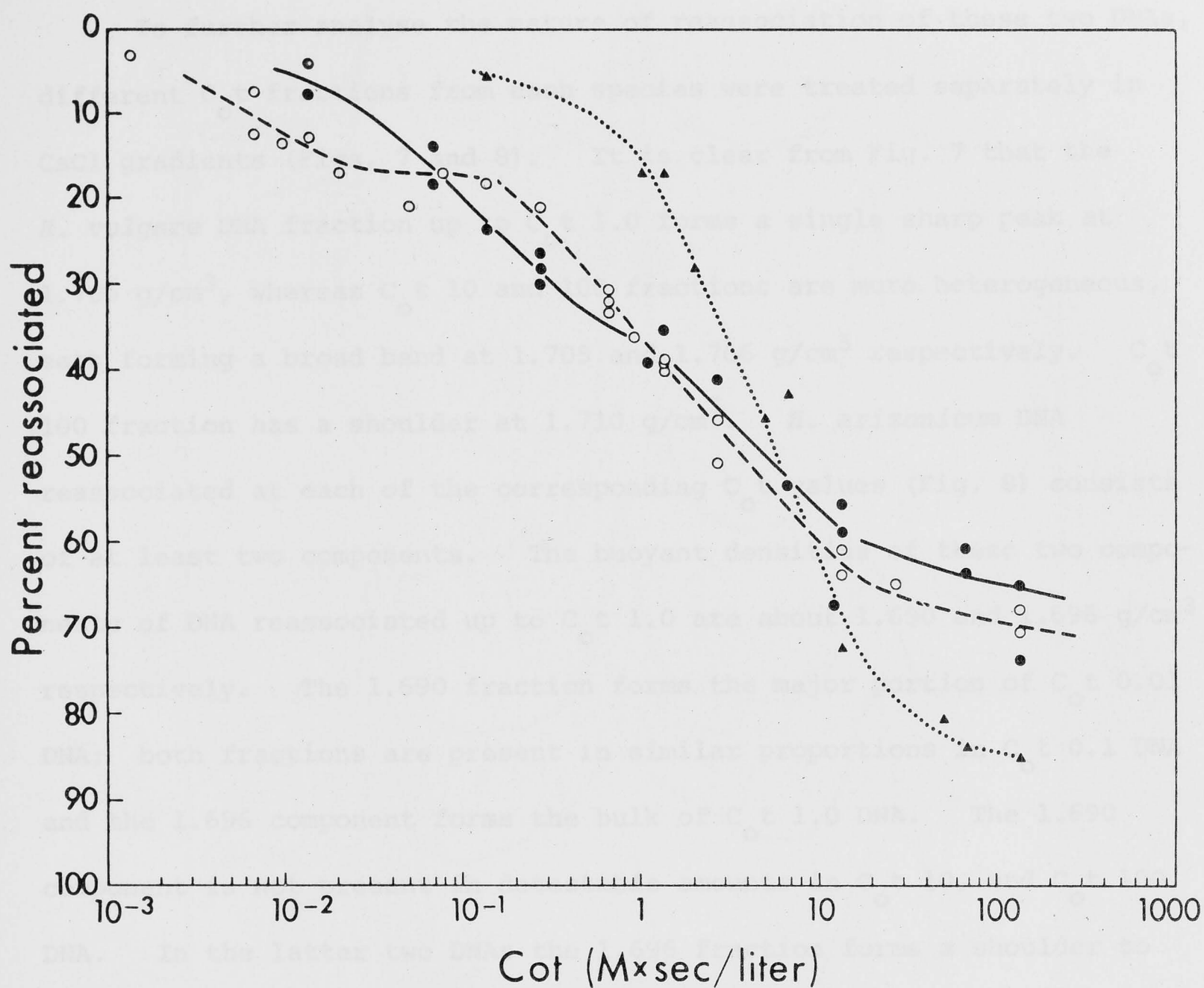


Fig. 6

Reassociation curves for *H. vulgare* (o - - o), *H. arizonicum* (●—●) and *E. coli* (▲.....▲) DNA in 0.12M PB, pH 6.8. DNA concentration ranged from 12.5 μ g to 500 μ g/ml depending on the C_0t value.

To further analyse the nature of reassociation of these two DNAs, different C_0t fractions from each species were treated separately in CsCl gradients (Figs. 7 and 8). It is clear from Fig. 7 that the *H. vulgare* DNA fraction up to C_0t 1.0 forms a single sharp peak at 1.705 g/cm³, whereas C_0t 10 and 100 fractions are more heterogeneous, each forming a broad band at 1.705 and 1.706 g/cm³ respectively. C_0t 100 fraction has a shoulder at 1.710 g/cm³. *H. arizonicum* DNA reassociated at each of the corresponding C_0t values (Fig. 8) consists of at least two components. The buoyant densities of these two components of DNA reassociated up to C_0t 1.0 are about 1.690 and 1.696 g/cm³ respectively. The 1.690 fraction forms the major portion of C_0t 0.01 DNA; both fractions are present in similar proportions in C_0t 0.1 DNA and the 1.696 component forms the bulk of C_0t 1.0 DNA. The 1.690 component is not present in detectable amounts in C_0t 10, and C_0t 100 DNA. In the latter two DNAs the 1.696 fraction forms a shoulder to the bulk of the DNA which has a density of about 1.706 g/cm³.

Thermal denaturation of DNA reassociated at different C_0t values was determined in 0.12M PB. Thermal denaturation profiles of C_0t 20 and C_0t 100 DNA of *H. vulgare* and C_0t 10 and C_0t 100 of *H. arizonicum* are presented in Figs. 9 and 10 respectively. The corresponding profiles for lower C_0t value DNA shows a very broad transition (not shown). C_0t 20 DNA of *H. vulgare* (Fig. 9) probably shows a monophasic transition (T_m = 76 C) but C_0t 100 DNA definitely has at least two components (T_m = 70.0 and 79.0). Both C_0t 10 and C_0t 100 DNA of *H. arizonicum* are clearly biphasic and the higher melting fraction has a T_m of about 78.5c. The T_m s of the lower melting fraction in C_0t 10 and C_0t 100 DNA are about 62.5 and 57.5 respectively. The transition of the lower melting fractions of C_0t 100 DNA starts earlier than that of C_0t 10 DNA.

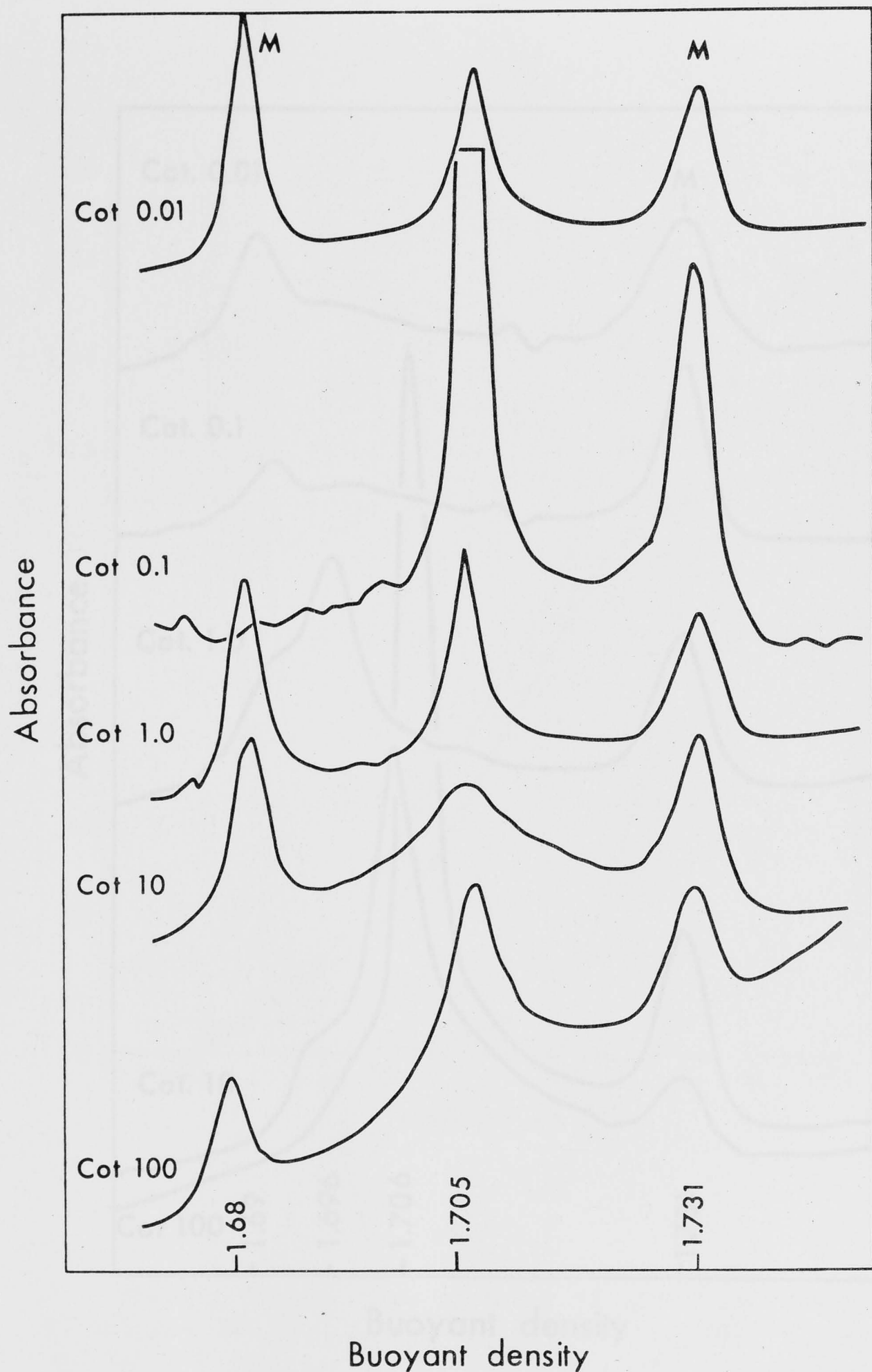


Fig. 7

Analytical ultracentrifugation (in CsCl) profiles of *H. vulgare* reassociated DNA fractions up to C_{ot} values indicated in the Figure. *Micrococcus luteus* DNA ($\rho = 1.731 \text{ g/cm}^3$) and poly (dA-dT) (1.68 g/cm^3) (except in C_{ot} 0.1) were used as markers in all cases. Variation in base line and seemingly relative amounts of marker are due to instrumentation.

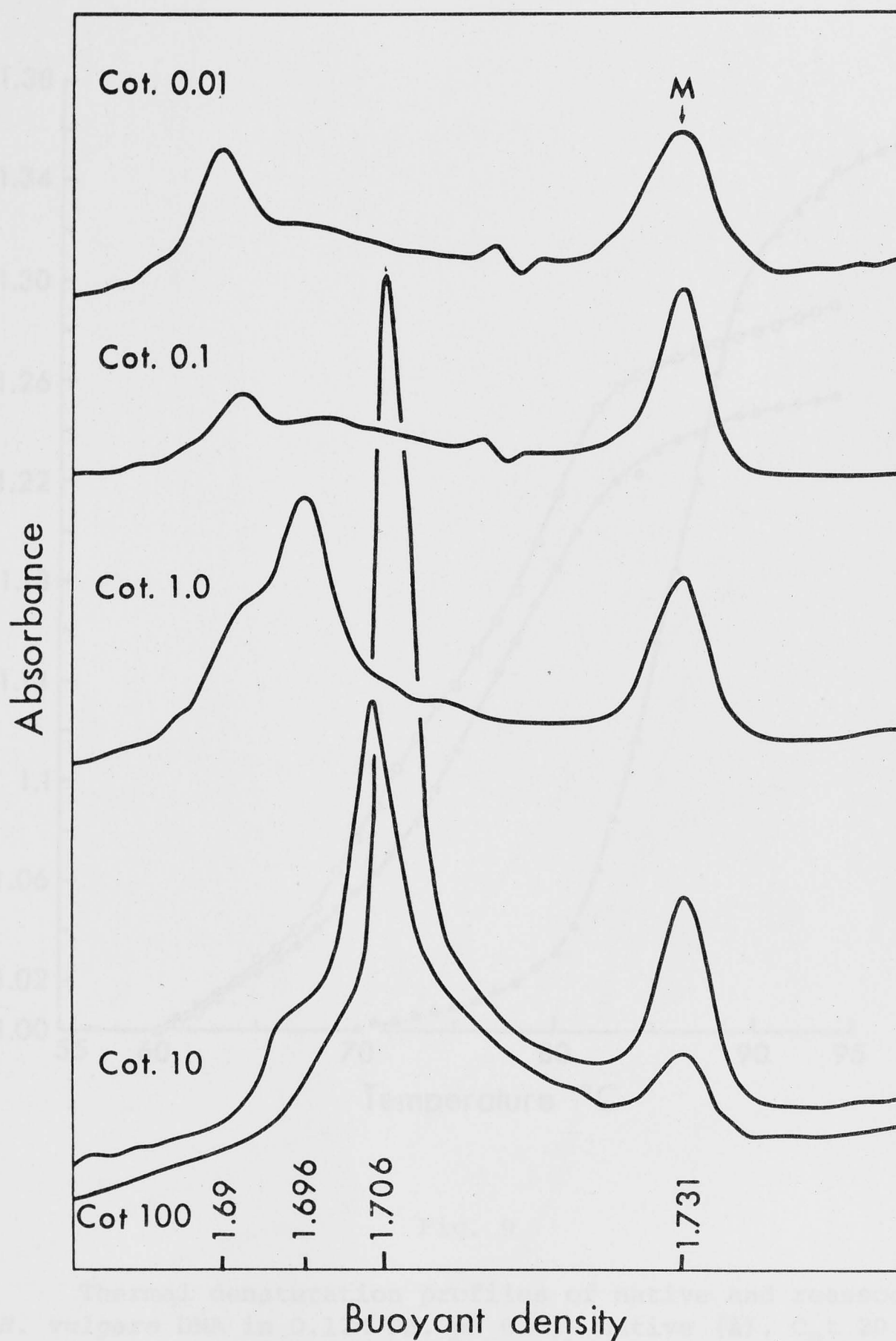


Fig. 8

UV scans of *H. arizonicum* reassociated DNA fractions (up to C_{ot} values indicated in the Figure) in neutral CsCl gradients. *Micrococcus luteus* DNA ($\rho = 1.731 \text{ g/cm}^3$) was used as a marker in each case. Variation in relative amounts of marker is due to instrumentation.

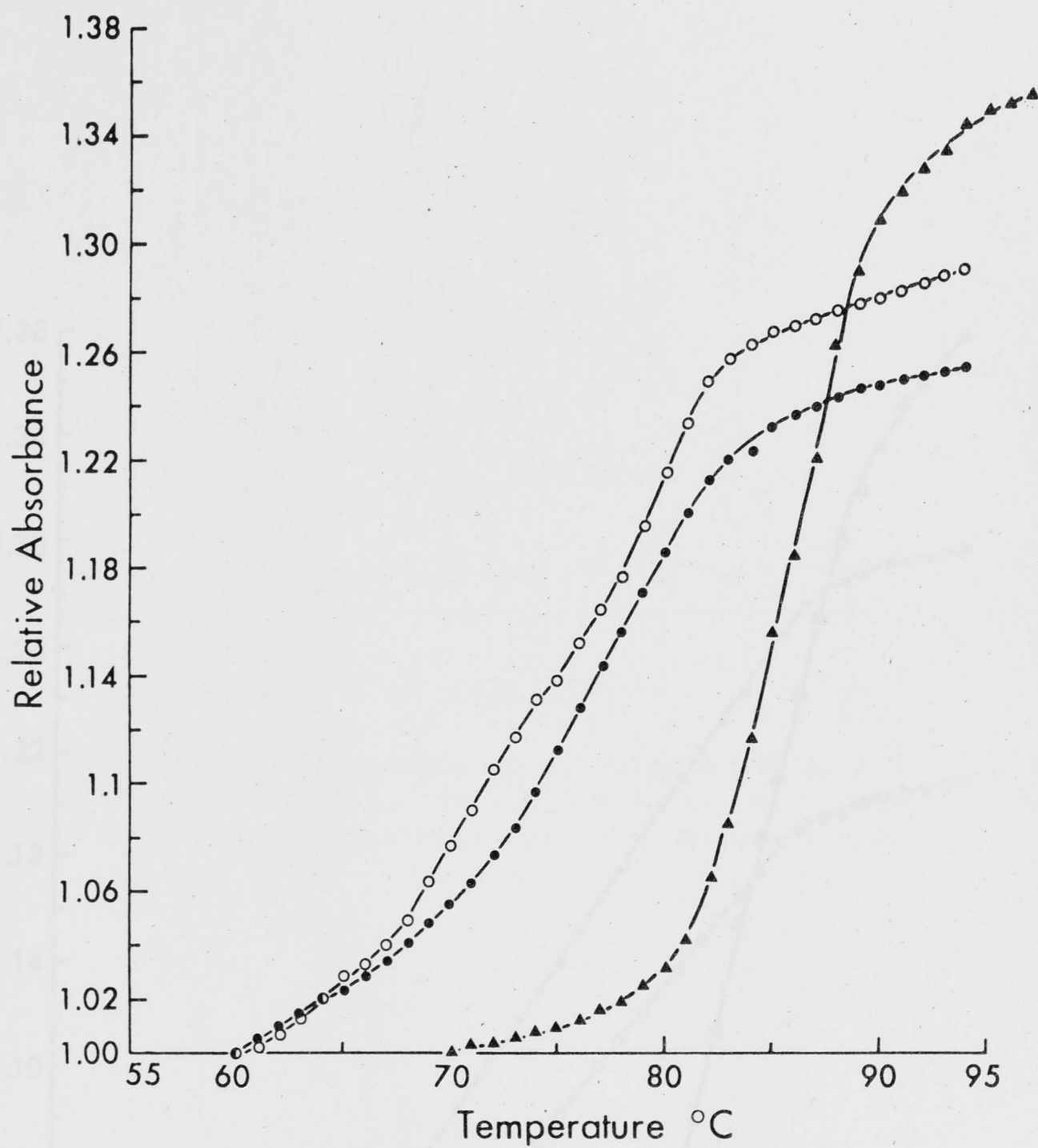


Fig. 9

Thermal denaturation profiles of native and reassociated *H. vulgare* DNA in 0.12M PB, pH 6.8; native (▲), C₀t 20 (●) and C₀t 100 (○).

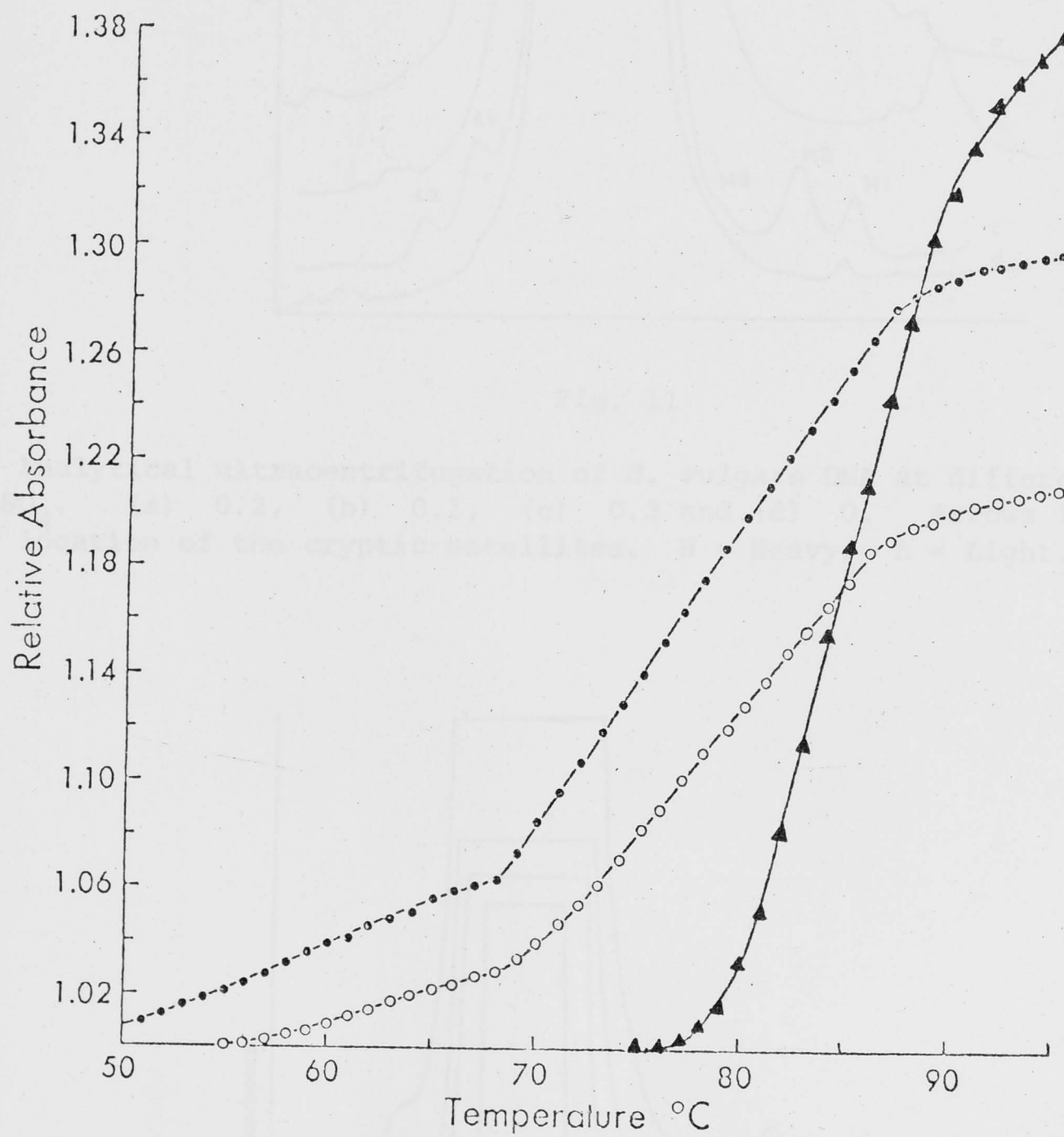


Fig. 10

Thermal denaturation profiles of native and reassociated *H. arizonicum* DNA in 0.12M PB, pH 6.8; native (▲), C_0t 10 (○) and C_0t 100 (●).

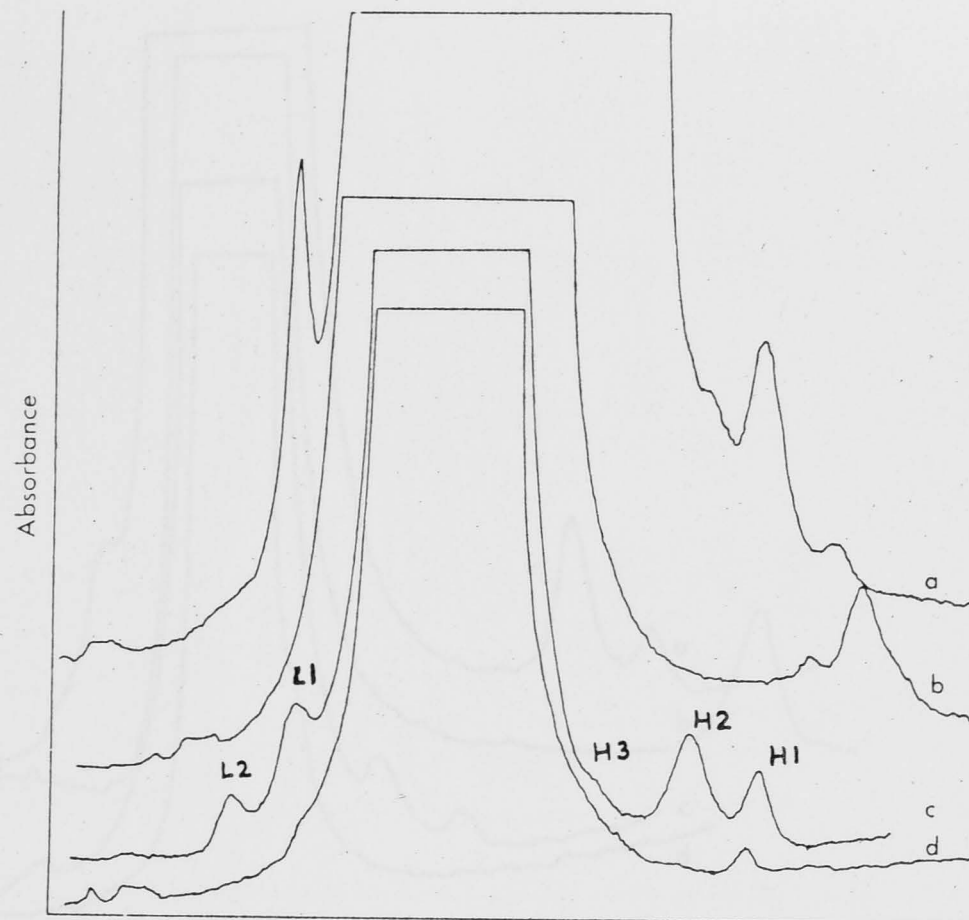


Fig. 11

Analytical ultracentrifugation of *H. vulgare* DNA at different R_F 's in Cs_2SO_4 . (a) 0.2, (b) 0.1, (c) 0.3 and (d) 0. Arrows indicate the location of the cryptic satellites. H = Heavy; L = Light.

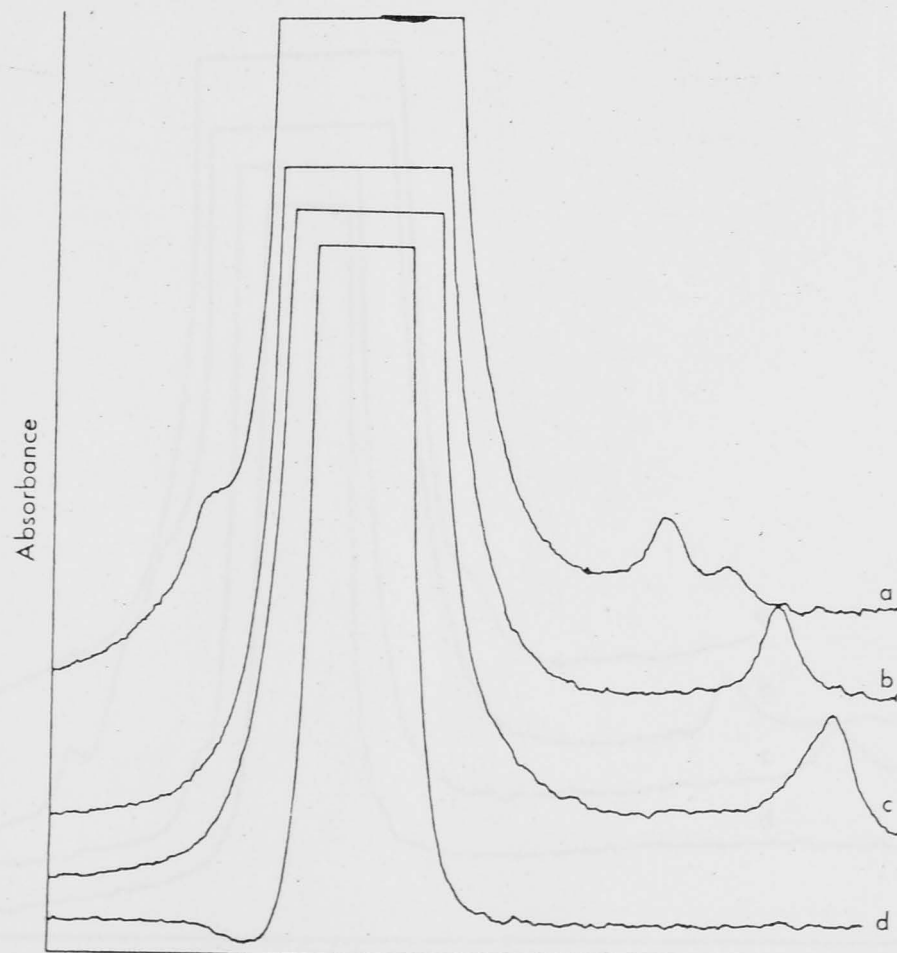


Fig. 12

Analytical ultracentrifugation of *H. agriocrithon* DNA at different R_F 's in Cs_2SO_4 . R_F 's (a) 0.3; (b) 0.2; (c) .1; (d) 0.

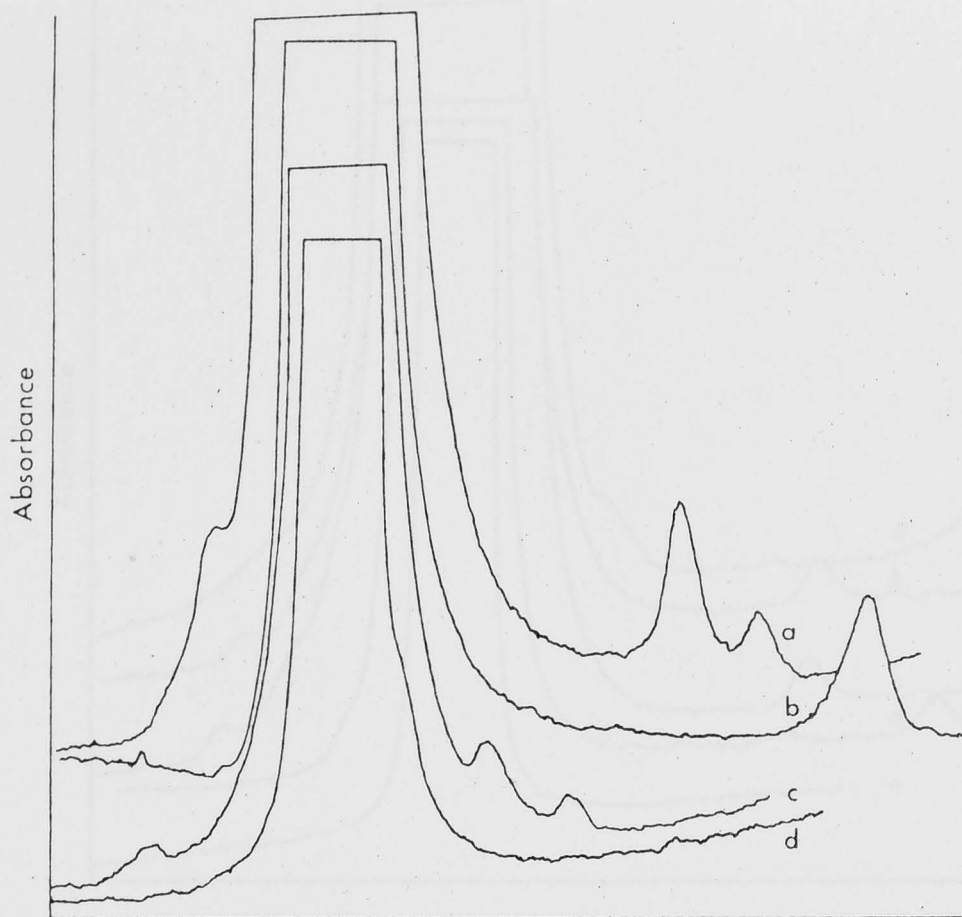


Fig. 13

Analytical ultracentrifugation of *H. spontaneum* DNA at different R_F 's in Cs_2SO_4 . R_F 's (a) 0.2; (b) 0.1; (c) 0.3; (d) 0.

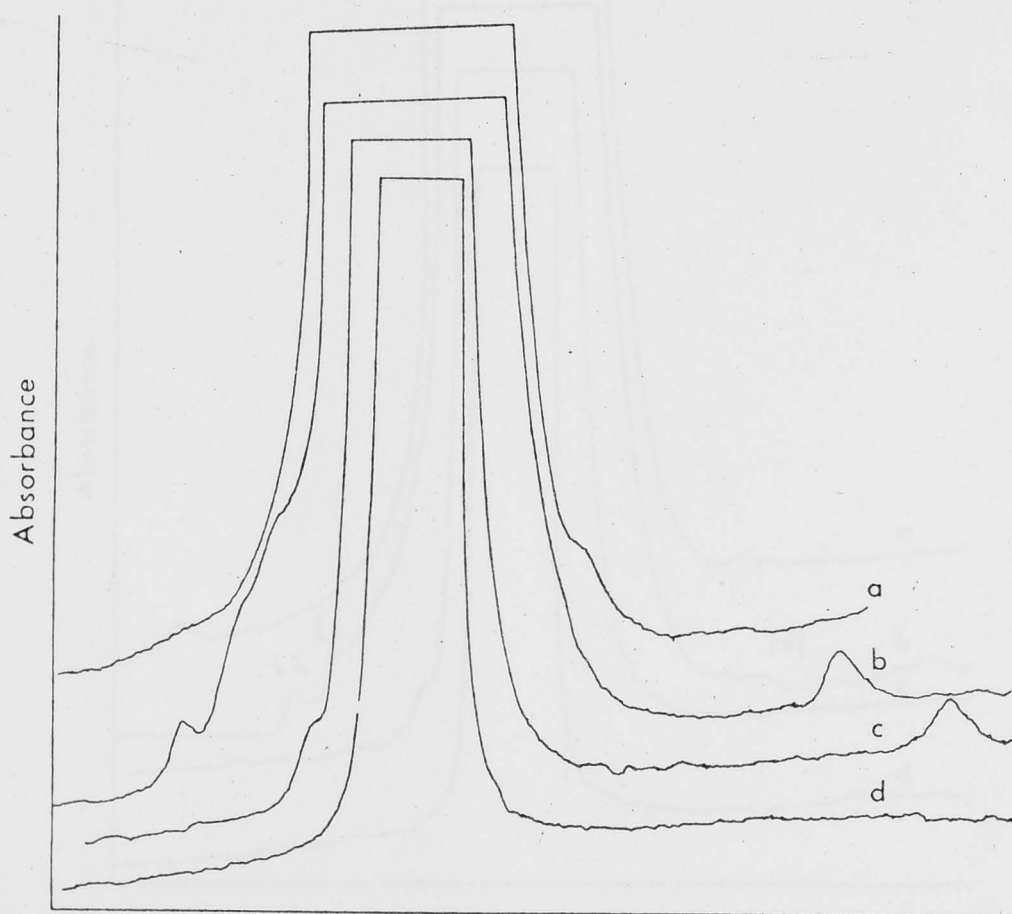


Fig. 14

Analytical ultracentrifugation of *H. californicum* (2x) DNA at different R_F 's in Cs_2SO_4 . R_F 's (a) 0.3; (b) 0.2; (c) 0.1; (d) 0.

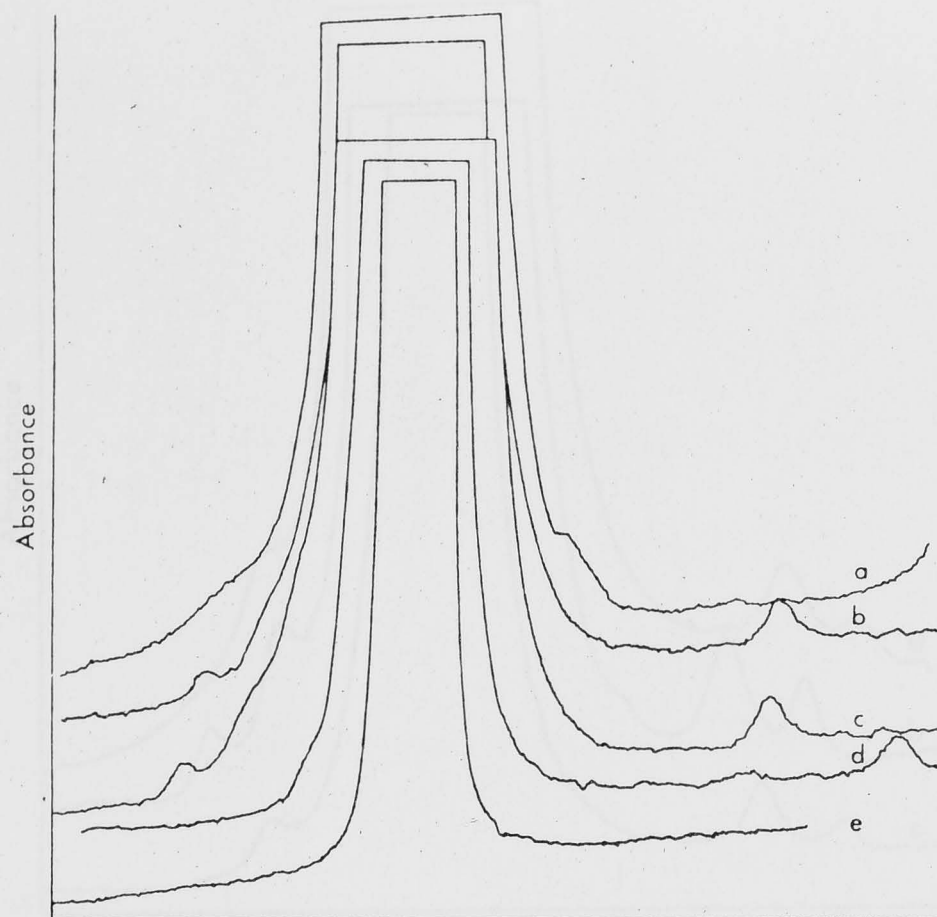


Fig. 15

Analytical ultracentrifugation of *H. jubatum* (4x) DNA at different R_F 's in Cs_2SO_4 . R_F 's (a) 0.4; (b) 0.3; (c) 0.2; (d) 0.1; (e) 0.

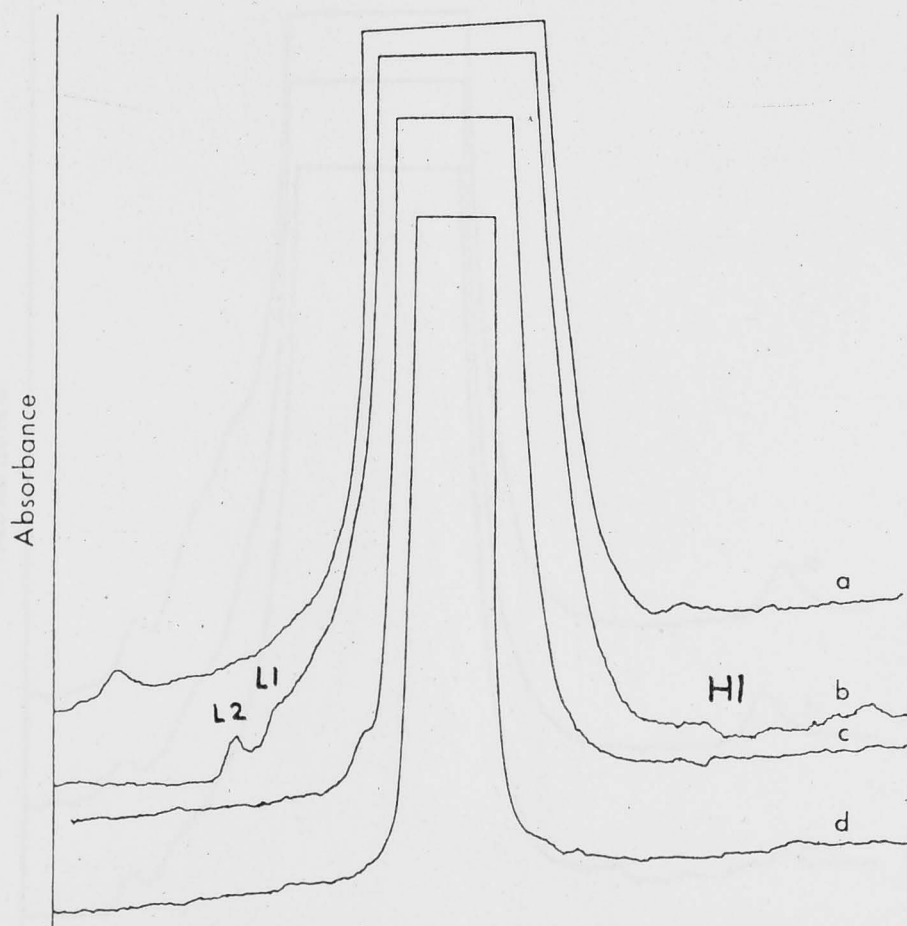


Fig. 16

Analytical ultracentrifugation of *H. arizonicum* (6x) DNA at different R_F 's in Cs_2SO_4 . R_F 's (a) 0.3; (b) 0.2; (c) 0.1; (d) 0. Arrows indicate the location of cryptic satellites. H = Heavy; L = Light.

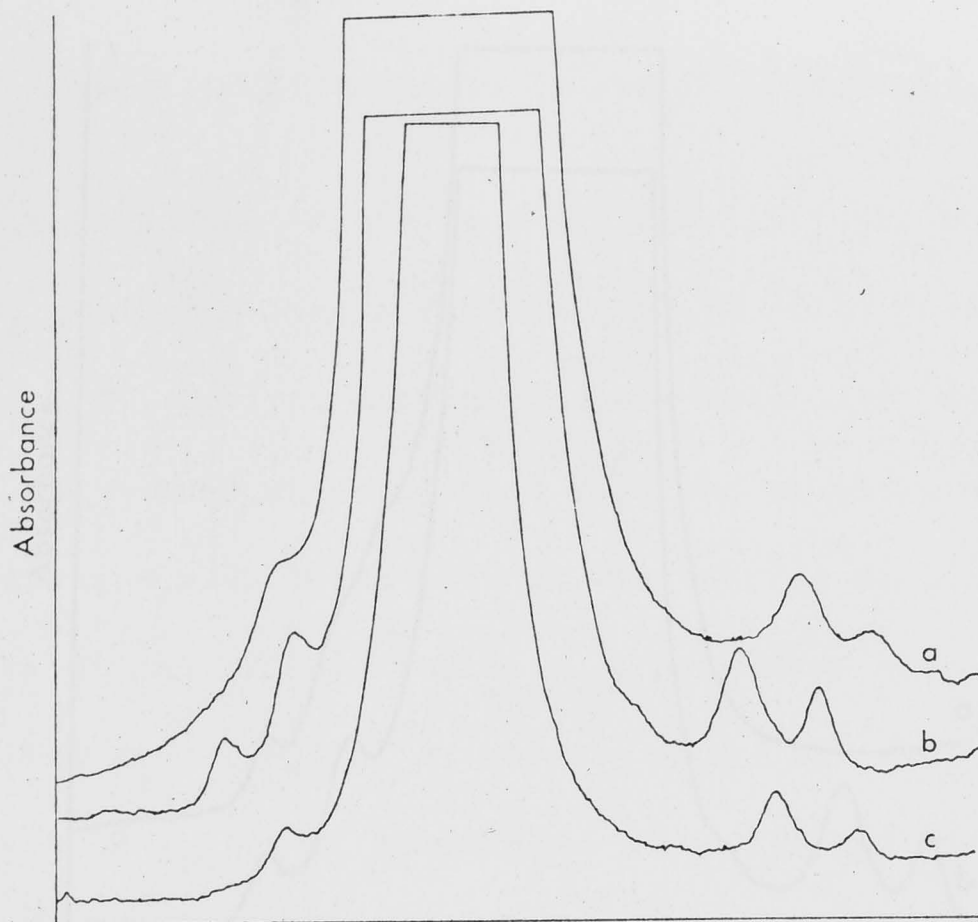


Fig. 17

Comparison of analytical ultracentrifugation profiles of DNA in $\text{Ag}^+ - \text{Cs}_2\text{SO}_4$ among related diploid species, (a) *H. agriocrithon* (R_F 0.3); (b) *H. vulgare* (R_F 0.3) and (c) *H. spontaneum* (R_F 0.25).

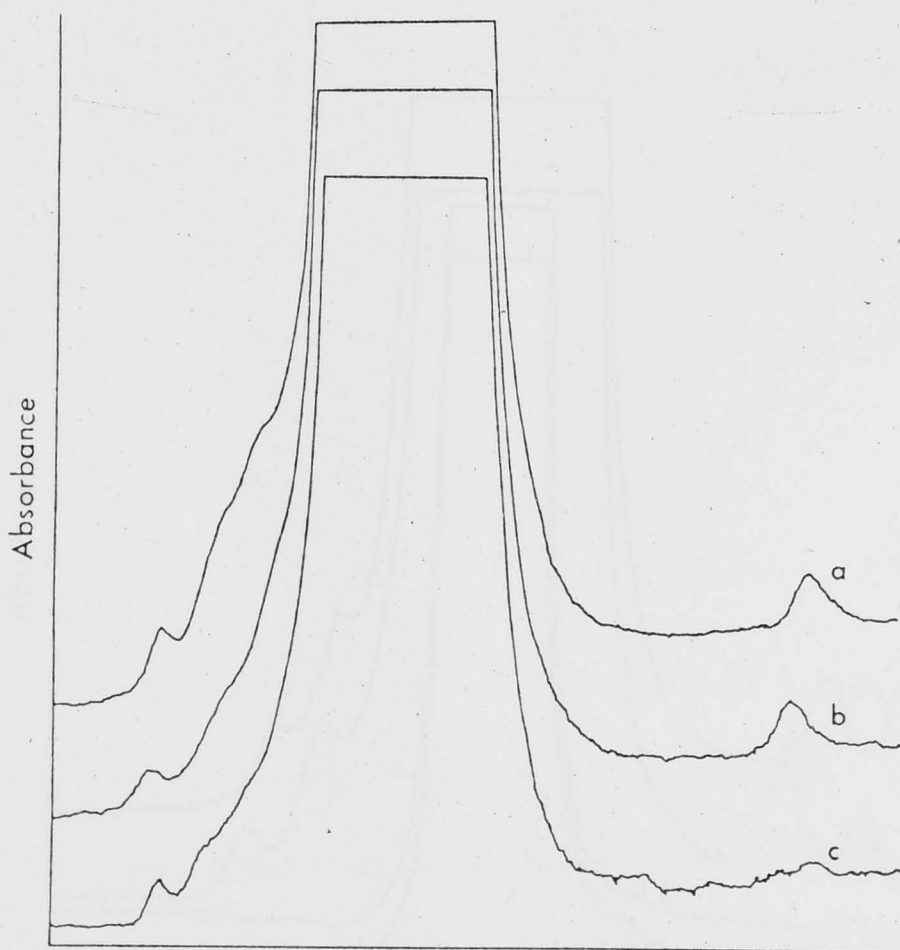


Fig. 18

Comparison of analytical ultracentrifugation profiles of DNA in $\text{Ag}^+ \text{Cs}_2\text{SO}_4$ among the members of the polyploid series. $R_F = 0.2$; (a) *H. californicum* (2x); (b) *H. jubatum* (4x); and (c) *H. arizonicum* (6x).

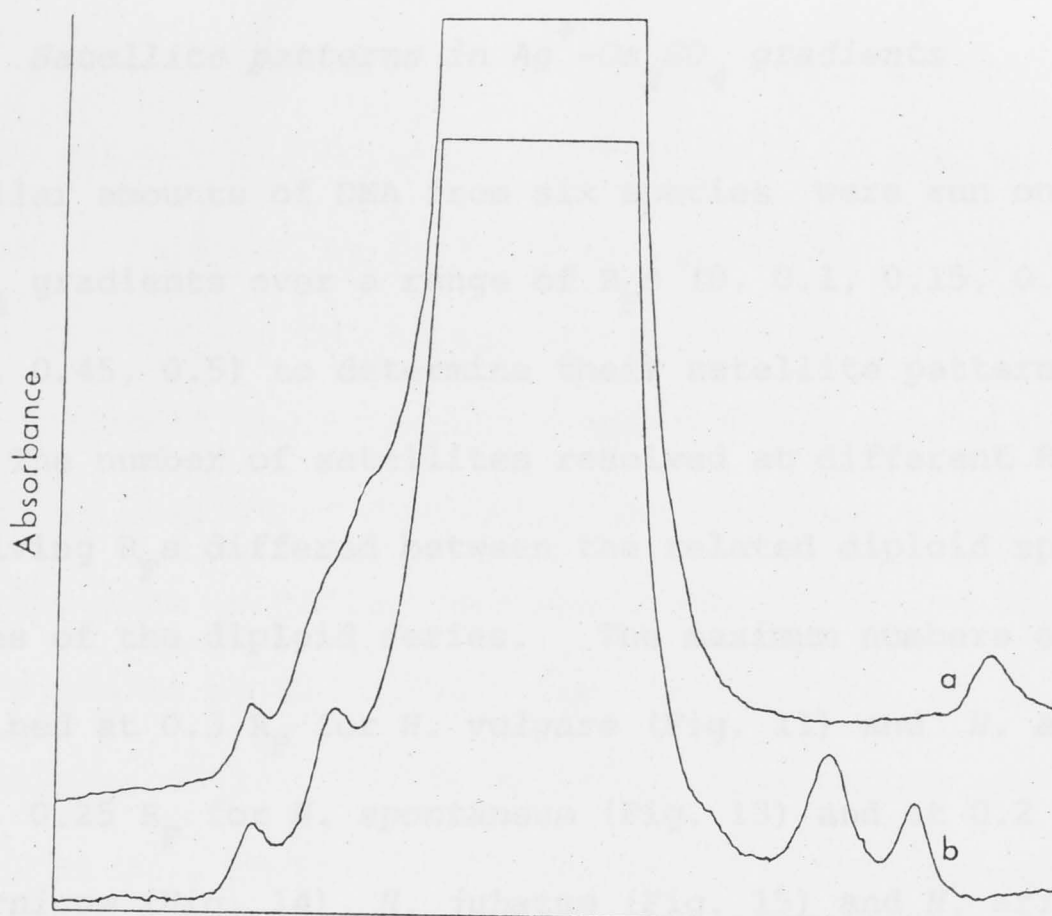


Fig. 19

Comparison of analytical ultracentrifugation profiles of DNA in $\text{Ag}^+ - \text{Cs}_2\text{SO}_4$ of (a) *H. californicum* R_F 0.2; (b) *H. vulgare* R_F 0.3.

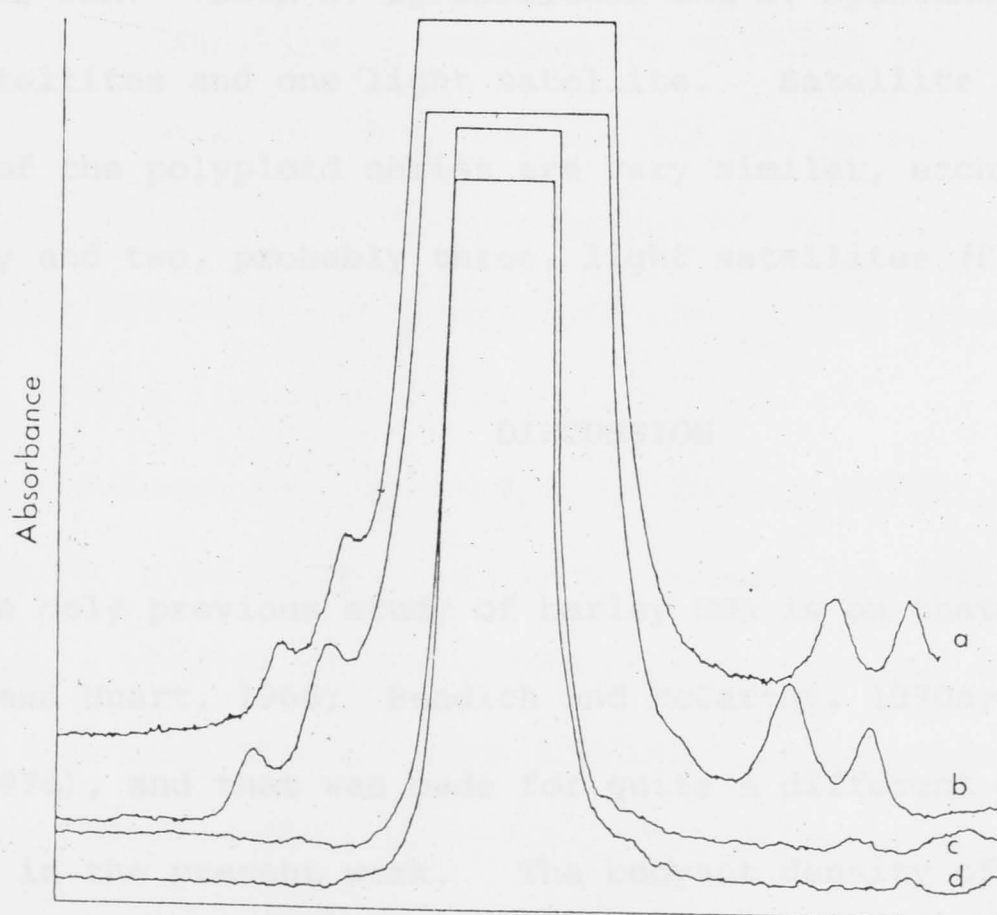


Fig. 20

Analytical ultracentrifugation in Cs_2SO_4 of *H. vulgare* DNA (b and c) and *H. vulgare* plus B1 DNA (a and d). R_F 0.3 (a) and (b). R_F 0 (c) and (d).

Satellite patterns in $Ag^+ - Cs_2SO_4$ gradients

Similar amounts of DNA from six species were run on analytical $Ag^+ - Cs_2SO_4$ gradients over a range of R_F s (0, 0.1, 0.15, 0.2, 0.25, 0.3, 0.35, 0.4, 0.45, 0.5) to determine their satellite patterns (Figures 11-19). The number of satellites resolved at different R_F s and the best resolving R_F s differed between the related diploid species and the species of the diploid series. The maximum numbers of satellites were obtained at 0.3 R_F for *H. vulgare* (Fig. 11) and *H. agriocrithon* (Fig. 12), 0.25 R_F for *H. spontaneum* (Fig. 13) and at 0.2 R_F for *H. californicum* (Fig. 14), *H. jubatum* (Fig. 15) and *H. arizonicum* (Fig. 16). The best resolved pattern of each species was used for a comparison between related diploid species (Fig. 17), species of the polyploid series (Fig. 18) and *H. vulgare* and *H. californicum* (Fig. 19). At higher R_F s (above 0.3) the resolution gradually disappeared. At 0.3 R_F , three heavy and two light satellites were resolved in *H. vulgare* DNA. Both *H. agriocrithon* and *H. spontaneum* showed two heavy satellites and one light satellite. Satellite patterns in the members of the polyploid series are very similar, each species showing one heavy and two, probably three, light satellites (Fig. 18).

DISCUSSION

The only previous study of barley DNA is on that of *H. vulgare* (Ledoux and Huart, 1968; Bendich and McCarthy, 1970a; Ranjekar et al. 1976), and that was made for quite a different purpose than that intended in the present work. The buoyant density of *H. vulgare* ($1.700 \pm 0.001 \text{ g/cm}^3$) now obtained is in close agreement with the 1.701 reported by Bendich and McCarthy (1970a), though somewhat lower than the value (1.702) reported by Ranjekar et al. (1976) and Ledoux

and Huart (1968). The T_m of 85.8 C is also very similar to that obtained by Bendich and McCarthy (1970a; 85.2 C) but lower than the value 87 C reported by Ranjekar et al. (1976). It is evident from Table II that the DNA of the six species of *Hordeum* are very similar in terms of their buoyant densities and melting temperatures. This result is consistent with other studies where these two parameters do not seem to differ much among species within the same genus (*Neurospora*, Dutta et al., 1976; *Zea*, Levings III, et al., 1976; *Allium*, Ranjekar et al., 1978b; *Hordeum*, Subrahmanyam and Azad, 1978; Chakrabarti et al., 1978b). The similarity of the buoyant densities and melting temperatures of different species of *Hordeum* presumably reflect the similarity of the base composition of their DNAs.

Reassociation studies on *H. vulgare* DNA have produced a monophasic curve similar to that of wheat (Ranjekar et al., 1976). In the present study a biphasic reassociation curve was obtained but there is no conflict with Ranjekar's result because the difference is due to DNA reassociating between $C_0 t 10^{-3}$ and 10^{-2} , which was not examined by Ranjekar et al. (1976) who did not go below $C_0 t 10^{-2}$. There were differences in the percentage of DNA reassociating up to $C_0 t 100$, the main difference being in the rapidly reassociating fractions (Table III).

The reassociation curve of *H. arizonicum* is different to that of *H. vulgare*, especially at lower $C_0 t$ values (Fig. 6). This is not surprising in view of the fact that repeated DNA (as well as single copy DNA) may have diverged independently among species within a genus (*Lathyrus*, Narayan and Rees, 1977). The point is strengthened by the observation that DNA of *H. arizonicum*, reassociated at different $C_0 t$ values, always shows at least two components in isopycnic CsCl gradients. The buoyant densities of these two components suggest that they are relatively AT-rich. DNA reassociated at $C_0 t 10$ and 100 is

composed of base sequences which are similar to those of *H. vulgare* judged by the buoyant densities. The thermal transition of reassociated DNA ($C_0 t$ 10 and $C_0 t$ 100 fractions of *H. arizonicum* and $C_0 t$ 100 fractions of *H. vulgare*) supports a two component system, as observed in CsCl. An additional piece of evidence supporting the conclusion that repeated fractions of *H. arizonicum* are indeed different from those of *H. vulgare* will be found in the study of their respective satellite DNAs (Chapter IV).

In the $Ag^+-Cs_2SO_4$ analysis the related diploid species differed from the species of the polyploid series in respect of the R_F for best resolution and the number of satellites formed. Whilst two satellites on the heavy side of the mainband were common to *H. vulgare*, *H. argiocrithon* and *H. spontaneum*, only one was seen in the polyploid series. For *H. vulgare*, Ranjekar (1976) and Deumling (1976) observed two heavy satellites. In *H. vulgare* a third heavy satellite was observed in the present study. Whereas Deumling et al. (1976) observed one light satellite, two were found in the present work. Two, probably three, light side satellites were observed in DNAs from *H. californicum*, *H. jubatum* and *H. arizonicum*, but the amounts gradually diminished with increase in ploidy (Fig. 18). This gradual decrease in their relative amounts can be attributed either to the dilution caused by the presence of a second genome (contributed by *H. compressum*, Starks and Tai, 1974) in *H. jubatum*, and a third genome from another unassigned species in *H. arizonicum*, or else because each progenitor species had similar satellites for which modulation might have resulted in reduced amounts. Further experiments with *H. compressum* would be required to rule out one or the other alternatives or whether a combination of these is involved.

The three species of the polyploid series differ from the three species of the diploid series in having one rather than two heavy satellites. The question of whether the lone heavy satellite is

conserved will be discussed in Chapter IV, which considers the isolation of satellites, their characterisation and localization on chromosomes.

As already pointed out (Chapter II, Chakrabarti et al., 1978a) the buoyant density ($1.700 \pm 0.001 \text{ g/cm}^3$) of DNA from one of the several barley associated bacteria (B1) coincides in neutral CsCl gradients with that of native barley DNA. It was estimated that less than 0.0015% of the DNA extracted from barley roots and then purified, could be due to the B1 bacterium. This possible contamination is an order of magnitude less for DNA from shoots, the material used in the present work, but grown under different conditions. Nonetheless, a reconstruction experiment was done to see whether B1 DNA could contribute to the satellite pattern either as an extra satellite or by changing the relative amounts of the satellites. The addition of up to 20% B1 DNA had no significant effect on the satellites observed using *H. vulgare* DNA (Fig. 20).

CHAPTER IV

SATELLITE DNAs OF *H. VULGARE* AND *H. ARIZONICUM*: ISOLATION, PHYSICAL CHARACTERIZATION AND LOCALISATION ON CHROMOSOMES

INTRODUCTION

It has been shown in Chapter II that all six species studied contain both light and heavy cryptic satellites. Although DNA satellites have been reported for several plants, very few have been characterised in detail. Thermal denaturation profiles of satellites from wheat (Ranjekar et al., 1978a), flax (Timmis and Ingle, 1977) and *Cymbidium* (Capesius, 1976) show monophasic transitions, whereas those of melon, (Bendich and Anderson, 1974; Bendich and Taylor, 1977; Sinclair et al., 1975), tomato (Chilton, 1975), cucumber (Timmis and Ingle, 1977) and *Tropaeolum* (Deumling and Nagl, 1978) are biphasic, indicating the presence of two components. Upon reassociation, however, all these satellites revealed the presence of two components.

Chromosomal localization of satellites have been reported for three species of plants, *Scilla* (Timmis et al., 1975), rye (Appels et al., 1978) and wheat (Peacock et al., 1977a and 1977b). In at least two of these (*Scilla* and rye) the satellites are localised in distal heterochromatic regions.

Peacock et al., (1977a) make passing reference to the isolation and localisation of one satellite from *H. vulgare* but no details were provided. In *H. vulgare* two satellites have been reported by Ranjekar et al., (1976). One of these was characterised in terms of thermal denaturation and reassociation (Ranjekar et al., 1978a) and revealed the presence of two components. A comparison of the

physical properties of one wheat satellite with one of the two satellites of barley revealed that they differ in thermal dissociation profiles and most probably in the kinetic complexities of the major fast reassociating DNA fractions (Ranjekar et al., 1978a). No comparative data concerning the physiochemical properties of satellite DNA from related species within the same genus is available. This Chapter describes the isolation and purification of all the satellites from *H. vulgare* and *H. arizonicum* identified in Chapter III. Purified satellite DNAs have been analyzed in terms of their physical properties and the location of the heavy satellites on the chromosomes has been identified by *in situ* hybridization.

MATERIALS AND METHODS

The sources of *H. vulgare* (2x) and *H. arizonicum* (6x) used are listed in the Materials and Methods sections (Chapter III).

DNA Extraction

The sources for DNA extraction is essentially similar to that described in Chapter II except that the ribonuclease step was omitted as advised by Marmur (1963) in cases where DNA is to be used as a primer for complementary RNA (cRNA) synthesis.

Preparative $\text{Ag}^+ - \text{Cs}_2\text{SO}_4$ gradient centrifugation

R_F s for best resolution were obtained by analytical $\text{Ag}^+ - \text{Cs}_2\text{SO}_4$ centrifugation (Chapter III); the optimum for *H. vulgare* was 0.3 and for *H. arizonicum* 0.2. Silver nitrate (10 mM) was added to DNA to achieve the desired R_F value, and the mixture gently shaken for 2 h;

Cs_2SO_4 was added to an initial density of 1.50 g/cm^3 . A sample was first run in an analytical ultracentrifuge to check the resolution and the remainder of the DNA was then used for preparative centrifugation in a Beckman L265B ultracentrifuge, using a 50 Ti fixed angle rotor for 60 h at 44000 rpm at 20 C. 350 μg of DNA was placed in each tube. Each gradient was fractionated with the aid of a Gilford 2580 density gradient scanner, while absorbance was recorded by a Gilford 2400 spectrophotometer. Figs. 1 and 14 show typical UV profiles of such fractionation. Fractions corresponding to each satellite were chosen in such a way as to reduce contamination from adjoining peak(s). This meant that a portion of the satellite(s) had to be sacrificed. Fractions corresponding to each satellite from several gradients were pooled and samples were run in an analytical ultracentrifuge to check for purity. The preparative step was then repeated except that the initial density of Cs_2SO_4 was the same as the pooled fractions and no adjustment was made. UV profiles of the second preparative gradients are also shown in Figs. 1 and 14. A third preparative step was necessary for the purification of some satellites (H3, L1 and L2, Fig. 1; L1 and L2, Fig. 14). Fractions containing pure satellites were pooled, dialysed extensively against SSC (0.15M NaCl, 0.015M Na-citrate, pH 7.0) and then stored at -10 C.

Buoyant Density

DNA in SSC was mixed with CsCl to an initial density of 1.705 g/cm^3 and analysed in a Beckman Model E analytical ultracentrifuge (44000 rpm, 20 C, 18 h). Buoyant density was calculated with reference to samples of standard DNAs, either *Micrococcus luteus* or poly(dA-dT) or both.

Thermal Dissociation

With the exception of the H2 of *H. vulgare*, thermal dissociation of satellite DNA was carried out with a Gilford 250 Spectrophotometer equipped with a Gilford 2527 thermoprogrammer, 6046 analog multiplexer and 6051 recorder. DNA solutions in 0.12M phosphate buffer (0.18M Na⁺, pH 6.8, 0.3 ml) were added to cuvettes and a tapered teflon stopper was firmly placed over the cuvettes to prevent evaporation. The temperature of the cuvette chambers was increased by 1 C/min and both temperature and hyperchromicity were recorded. The T_m of *E. coli* DNA was determined for each experiment and was used for correction taking 90.5 C as the standard.

The amount of *H. vulgare* H2 satellite recovered after purification was too low for use in a Gilford 250 and its thermal dissociation was determined using a Zeiss spectrophotometer as described in Chapter III. Derivatives of increase in hyperchromicity ($\Delta^H/\Delta T$) were calculated by using a Digital Docwriter II computer and were plotted against temperature by a Hewlett-Packard 7200A graphic plotter. Such derivatives were employed in the differential analysis of denaturation data.

Reassociation

The denatured DNAs (100 C for 5 min) were allowed to reassociate at the appropriate temperature (T_m - 25 C) for 16 h (C₀t of about 4).

Synthesis of Complementary RNA

Satellite DNA (100 μ l, 3-4 μ g) in TE (10 mM Tris, 1 mM EDTA, pH 8.4) was denatured by heating for 5 min in a boiling water bath.

H^3 -Nucleoside triphosphates (UTP, CTP, GTP and ATP [each 100 μ l, 1 mCi/ml; specific activity > 10 Ci/mmol, Amersham]) were mixed in a tube and evaporated under vacuum. Denatured DNA (100 μ l in TE) was added to the tube. The solution was then adjusted to 40 mM Tris (pH 7.9), 10 mM $MgCl_2$, 160 mM KCl, 0.2 mM dithiothreitol, and 1 mM ATP (by adding cold ATP). *Escherichia coli* RNA polymerase (10 units, Sigma, USA) was added and the reaction allowed to proceed at 37 C monitoring polymerisation (incorporation of label in TCA precipitable material) at appropriate time points. A typical polymerisation reaction is presented in Fig. 2. Yeast carrier RNA (150 μ g, BDH) was added after 1 h and the reaction stopped by adding DNase (10 μ g; Worthington, DPF RNase-free). The mixture was left at room temperature (21 ± 1 C) for 30 min before adjusting it to a final concentration of 0.1% SDS and 10 mM EDTA. An equal volume of TE saturated phenol was added and the mixture gently stirred for 10 min and then centrifuged at 2000 rpm for 10 min. The aqueous phase was collected and the phenol layer re-extracted with distilled water (0.2 ml). The combined aqueous phases were then passed through a column (0.9 cm x 15 cm) of G-75 Sephadex (equilibrated with 0.1 x SSC) to separate unincorporated H^3 nucleotides from the H^3 c-RNA. Fractions (about 0.5 ml each) were collected and all label in the exclusion volume was found to be in the c-RNA (TCA precipitable material, Fig. 3). Fractions containing c-RNA were pooled and concentrated by evaporation under vacuum. The solution was made to 6 x SSC and stored at -20 C. Radioactivity was counted in a Beckman LS250 scintillation counter using a toluene based scintillant (0.5% w/v PPO in toluene).

In situ hybridization

(a) Preparation of plant materials

Root tips were collected in distilled water (0 C) and left over-

night at 0 C, then fixed in acetic acid:ethanol (1:3) for a minimum of 1 h at 21 ± 1 C (room temperature). The fixative was removed and the root tips washed in distilled water and treated with 1N HCl at 60 C for 5 min. After removal of HCl, 2% cellulysin (in water) was added and the mixture incubated at 37 C for 10 min. After washing with water, the root tips were put in to 45% acetic acid and squashed. The preparation was scanned under phase contrast to check the spread of chromosomes. The slide was then put on dry ice for at least 15 min, or dipped in liquid N_2 , and the coverslip removed with a scalpel blade. The slides were passed through two changes of absolute ethanol for 5 min each and finally air dried. The slides were stored in clean boxes.

(b) Hybridization procedure

Preparations were dehydrated in absolute ethanol and air dried. Chromosomal DNA was denatured by incubating the slides in 0.2N HCl at 37 C for 30 min, followed by dehydration through an alcohol series (50%, 70% and 100% twice, 2 min each). Labelled c-RNA 6-7 μ l was placed on the preparation and covered with a clean coverslip. After sealing the edges of the coverslip with rubber solution, the slides were placed on a metal tray and incubated in a waterbath at 65 C for 6-7 h. After incubation, the slides were treated in the following manner:

- (i) washed twice in 3 x SSC at 45 C, 10 min each;
- (ii) washed twice in 2 x SSC at 45 C, 10 min each;
- (iii) transferred to RNase 10 μ g/ml in 2 x SSC at 37 C for 30 min.
- (iv) washed six times in 2 x SSC at room temperature (21 ± 1 C), 10 min each;

- (v) dehydrated through 70% ethanol and 100% ethanol twice, 2 min each and then air dried.

(c) Autoradiography

All steps were carried out under an Ilford safe light. Ilford K2 emulsion in gel form was heated at 43 C for 10 min. A 1:1 dilution was made with 2% glycerol kept at 43 C. The mixture was kept at 43 C for five min more and then the slides were dipped one at a time. The slides were then left on the bench in the dark for at least 2 h to dry. After drying, the slides were put in a black slide-box, sealed with black tape, and kept at 4 C for various exposure times (10 days to 2 months).

The autoradiographs were developed at 20 C for 3 min in a developer very similar to that of Kodak D19b, rinsed in water, to which a dash of developer had been added, and fixed in Ilford Hypam rapid fixer, diluted 1:4 with distilled water. The slides were washed in running water for 10 min and finally in distilled water. The slides were then air dried.

(d) Staining

The preparation was stained with 5% Gurr R66 Giemsa for 20 min followed by differentiation with 0.066M phosphate buffer (pH 6.8) for 4-5 min. The slides were then rinsed in distilled water and air dried.

RESULTS

(a) *H. vulgare* satellites

Buoyant density analysis

All five satellites reported from analytical ultracentrifugation studies (Chapter III) can be isolated in pure form by repeated preparative centrifugation (Fig. 1). Approximate percentages of satellite

to total DNA and their buoyant densities in CsCl, are presented in Table I. Each heavy satellite of *H. vulgare* forms a single peak in CsCl gradients, whereas one light satellite (L1) splits in two and the other (L2) into 4 components (Fig. 4). No attempt was made to purify the individual components of light satellites observed in CsCl gradients.

TABLE I

Buoyant densities of *Hordeum* satellites in CsCl and their approximate % of total DNA

	Satellite *	% ⁺	Number of components in CsCl	Density g/cm ³	% G + C [†] content of each component
<i>H. vulgare</i>	H1	0.86	1	1.700	41.6
	H2	1.88	1	1.727	69.2
	H3	-	1	1.699	40.1
	L1	2.30	2	(a) 1.697	38.2
				(b) 1.688	28.6
	L2	1.30	4	(a) 1.713	54.4
				(b) 1.706	47.6
				(c) 1.696	37.5
				(d) 1.685	25.7

* H refers to satellites on the heavy, and L refers to satellites on the light side of main band DNA.

+ Calculated by comparing the area of satellites (Fig. 11, Chapter III) to an area occupied by a known amount of *M. luteus* DNA in an analytical CsCl gradient (average of five measurements).

- Not estimated.

† Calculated from buoyant density according to Mandel et al. (1968).

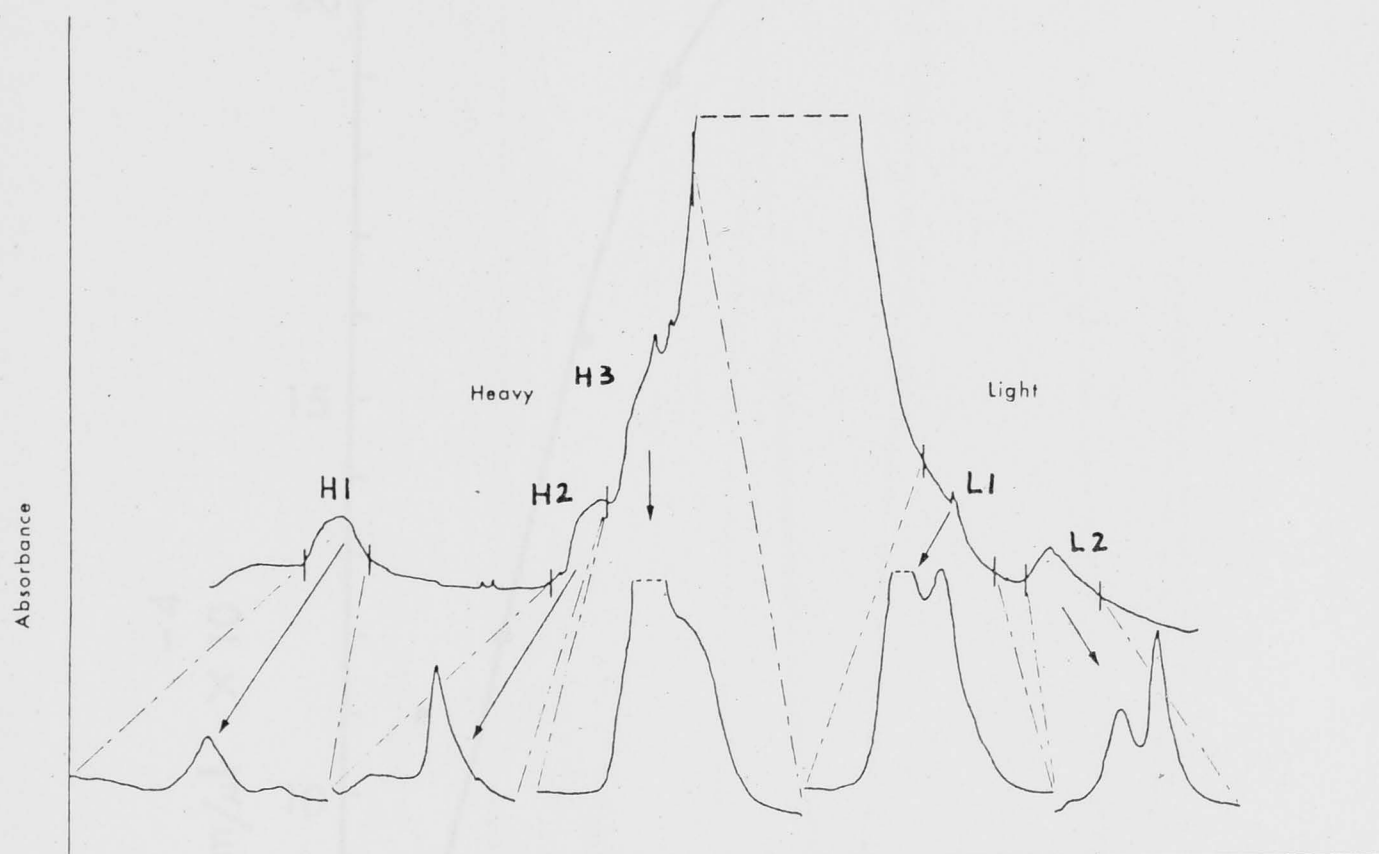


Fig. 1

UV absorbance profiles of *H. vulgare* DNA after preparative ultracentrifugation in Ag^+ - Cs_2SO_4 gradients. The upper figure shows a profile after the first preparative step. Fractions selected for each satellite are shown by vertical lines on the figures. Pooled fractions for each satellite from several gradients were centrifuged again and their absorbance profiles shown in the lower figures. Originally the lower figures were of the same scale as the upper one, but are shown here in reduced sizes. Where necessary a third preparative step was done.



Fig. 2

A typical curve showing progress of Ag^+ - Cs_2SO_4 ultracentrifugation of *H. vulgare* DNA. In this example, the Ag^+ - Cs_2SO_4 gradient was used as template.

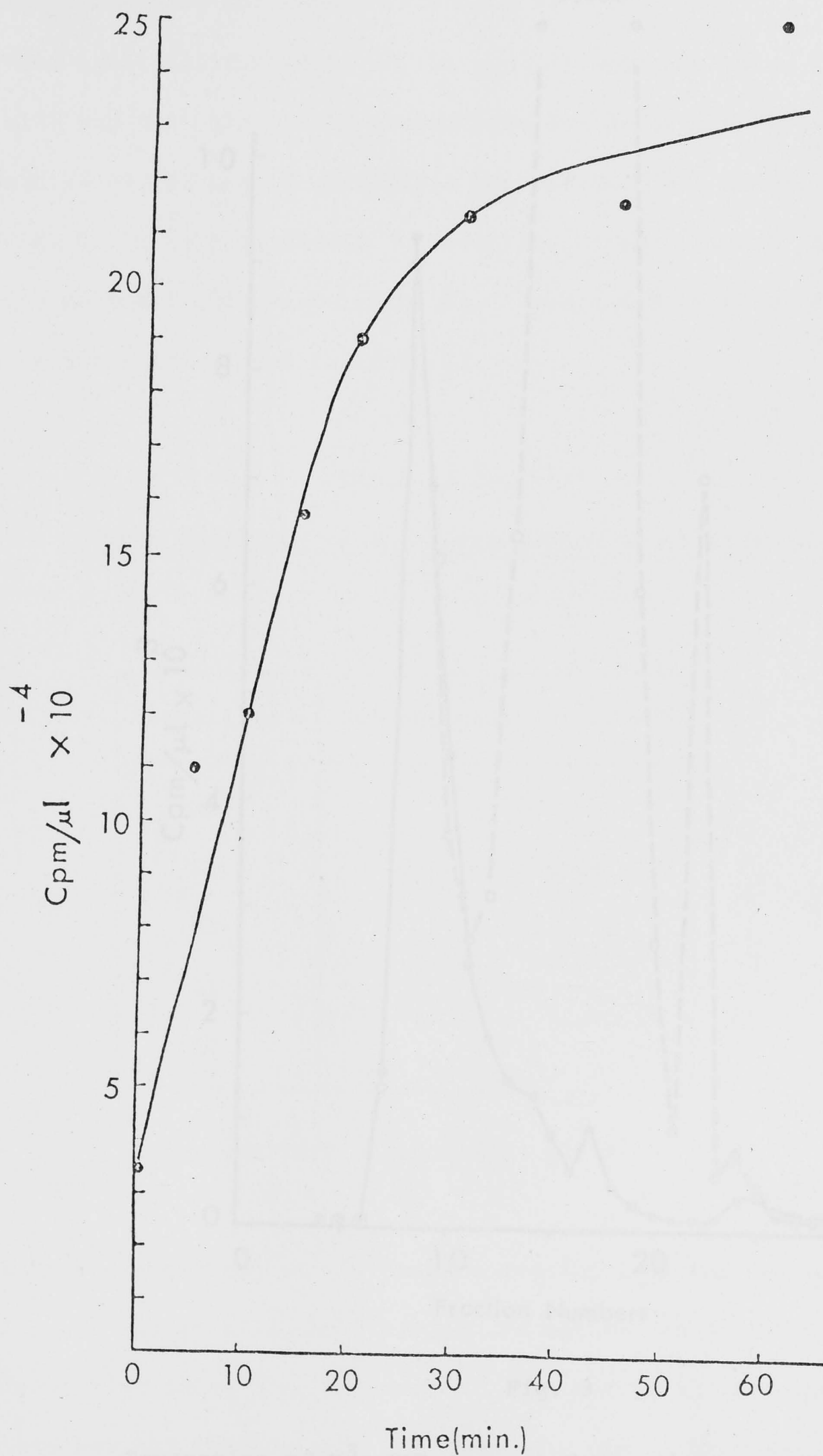


Fig. 2

A typical curve showing progress of H^3c -RNA synthesis (TCA precipitable material, 1 μ l sample). In this example, denatured DNA of the H1 satellite of *H. arizonicum* was used as template.

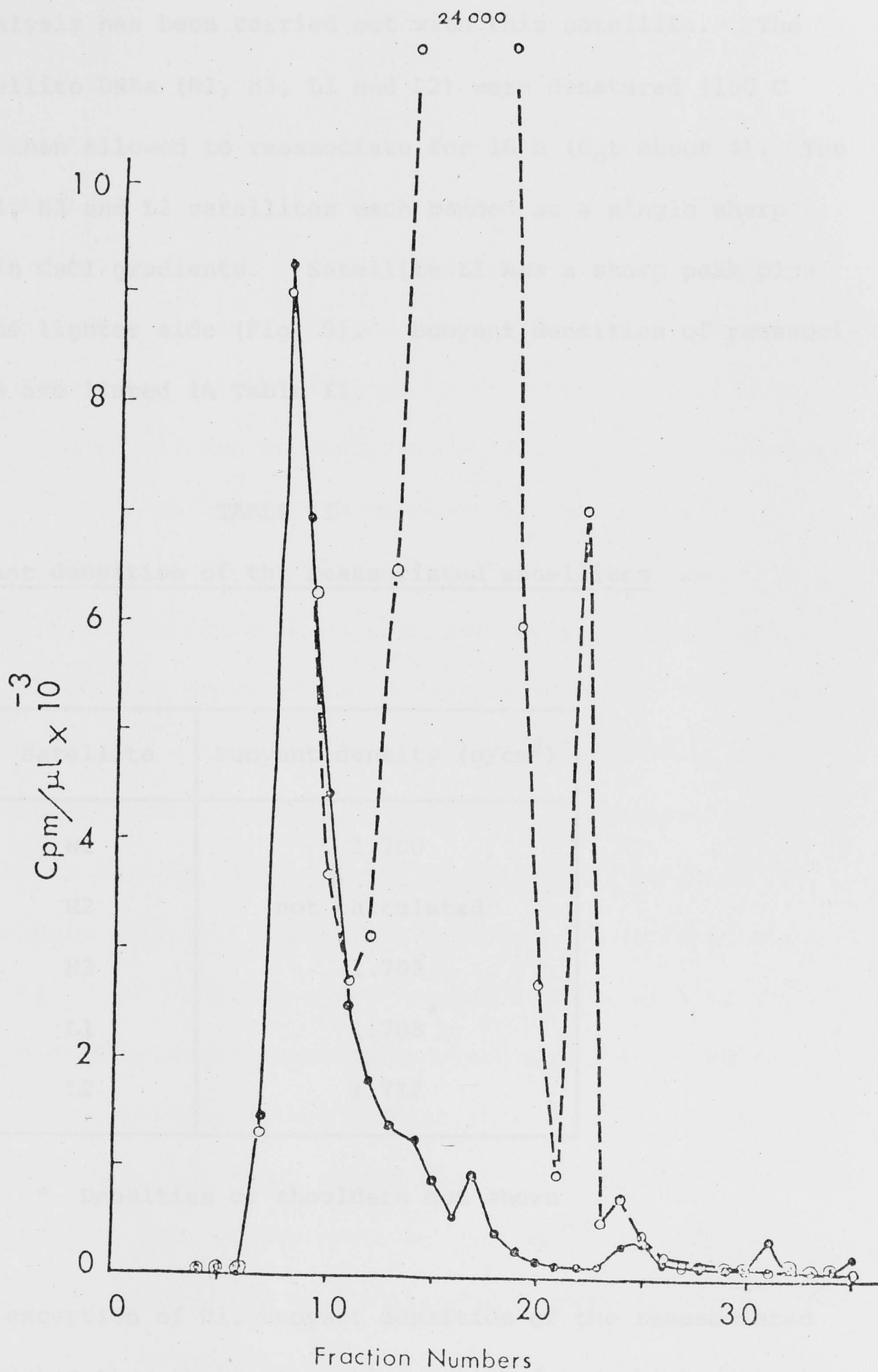


Fig. 3

Separation of H^3c -RNA (\bullet — \bullet) from unincorporated H^3 -nucleotides (o --- o) by fractionation through a Sephadex G-75 column. In this example the H^3c -RNA employed was that described in Fig. 2.

The amount of H2 recovered after purification was low, therefore, no detailed analysis has been carried out with this satellite. The other four satellite DNAs (H1, H3, L1 and L2) were denatured (100 C for 5 min) and then allowed to reassociate for 16 h (C_0t about 4). The reassociated H1, H3 and L2 satellites each banded as a single sharp peak (Fig. 5) in CsCl gradients. Satellite L1 has a sharp peak plus shoulders on the lighter side (Fig. 5). Buoyant densities of reassociated satellites are listed in Table II.

TABLE II

Buoyant densities of the reassociated satellites

Satellite	Buoyant density (g/cm ³)
H1	1.700
H2	not calculated
H3	1.703
L1	1.708 [*]
L2	1.712

* Densities of shoulders not shown

With the exception of H1, buoyant densities of the reassociated molecules are higher than their respective native forms which suggests some mismatching among base sequences. The native L2 DNA gave four components but the reassociated DNA gave only one. Whereas the native L1 DNA clearly resolved into two components, the reassociated DNA shows its heterogeneity in the form of shoulders to a major peak.

In alkaline CsCl each of the satellites shows a broad peak but no strand separation was observed suggesting that there is no strand bias of the satellite sequences.

Thermal dissociation profiles and T_m 's of native and reassociated satellite DNAs are presented in Figs. 6a-10a and Table III. Studies with DNAs of other organisms has revealed that an apparently simple thermal dissociation profile may show heterogeneity when the denaturation data are subjected to differential analysis (Vitek *et al.*, 1974; Lyubchenko *et al.*, 1976; Vizard and Ansevin, 1976). Thermal denaturation data of *H. vulgare* satellites was examined for heterogeneity (Figs. 6b-10b). For the native H3 DNA (Fig. 8a) it is obvious from the thermal transition profile that multicomponents are present and, in this example, there are probably two molecular species. This conclusion can be reached by examining the differential plot (Fig. 8b). In the case of native L1 DNA, it is more difficult to estimate the number of components from the denaturation profile (Fig. 9a), but differential analysis (Fig. 9b) reveals the presence of at least three thermal components. Differential analysis also assists with the estimation of accurate melting temperatures since these are read from peak values. In most multicomponent systems it is fairly easy to sketch in likely contributors which sum to the overall profile. It should be realised that some of the peaks in the overall profile are false, since they represent the sum of overlapping contributions. The T_m 's (Table III) have been calculated in this way and Fig. 17b provides an illustrating example of such a calculation. In general terms, though with the possible exception of H2 DNA, which was not analyzed as extensively (see Materials and Methods), all satellite DNAs contain more than one thermal component. Thermal transitions of all reassociated DNAs are broad and are more complex than the original melts, both

in having more components and having lower T_m 's.

The lowering of the T_m of reassociated DNA is caused by base mismatching (Bonner *et al.*, 1973; Ullman and McCarthy, 1973). Since native and reassociated satellites of *H. vulgare* are multicomponent it is difficult to estimate the % base mismatch (usually calculated from the difference in the T_m 's of native and reassociated DNA).

In situ hybridization

The chromosomes of *H. vulgare* are meta- or submetacentric in character. The nuclear location of the H1, H2 and H3 satellites is clear (Fig. 11) since H^3 -complementary RNA made from these satellites appears only in nuclei. Although attempts were made to hybridize H^3 -RNA complementary to H1, H2 and H3 satellites of *H. vulgare* to chromosome preparations of the same species, only H3 gave satisfactory results (Fig. 12). In the other cases (H1 and H2) the amount of label uptake was insufficient to allow confident interpretation of sites of localisation though from the few observations that were possible it appeared that H2 was located at some of the centromeres (Fig. 13).

All chromosomes of *H. vulgare* contain the H3 sequences in regions adjacent to centromeres but some chromosomes have the sequences on other parts as well (Fig. 12). It is not possible to construct a definite pattern of distribution of the satellite sequences in the karyotype because of insufficient data.

Light satellites do not show any hybridization at all even after two months exposure.

TABLE III

Melting temperature of native and reassociated satellite DNAs
of *H. vulgare*

Satellite	Native satellite		Reassociated satellite	
	T _m [*] native DNA	Thermal components (minimum) [*]	T _m [*] reassociated DNA	Thermal components (minimum)
H1	87 [†] , 93	2	71, 75.5, [†] 80.5 [†] , 85	4
H2	91 ^{**}	apparently 1	not done	not known
H3	83 [†] , 91.5	2	67, 71, 77 [†]	3
L1	86 [†] , 90,	3	80, 87, 92.5, 95.5 [†]	4
L2	86.5, 91, 95 [†]	3	87, 91, 95 [†]	3 plus

* Determined from differential plot after reconstruction (see Results).

** Determined from thermal denaturation profile.

† Major component (occupying maximum area under the curve).

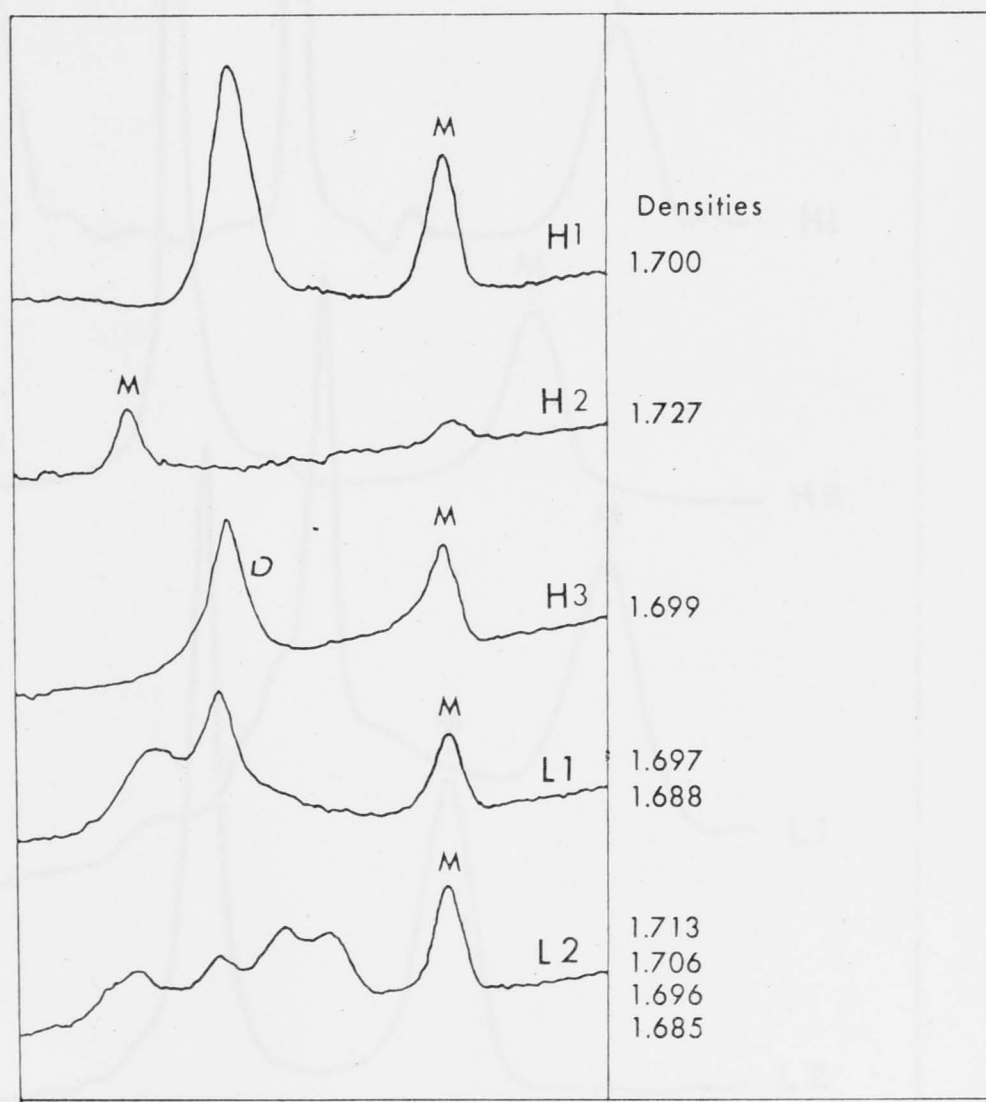


Fig. 4

UV absorbance profiles of native satellite DNAs of *H. vulgare* in analytical CsCl gradient (neutral). M refers to marker DNA usually *Micrococcus luteus* ($\rho = 1.731 \text{ g/cm}^3$) but for H2 poly(dA-dT) $\rho = 1.680 \text{ g/cm}^3$. Densities (g/cm^3) of individual components are shown to the right. H1, H2, H3, L1, and L2 refer to satellite numbers.

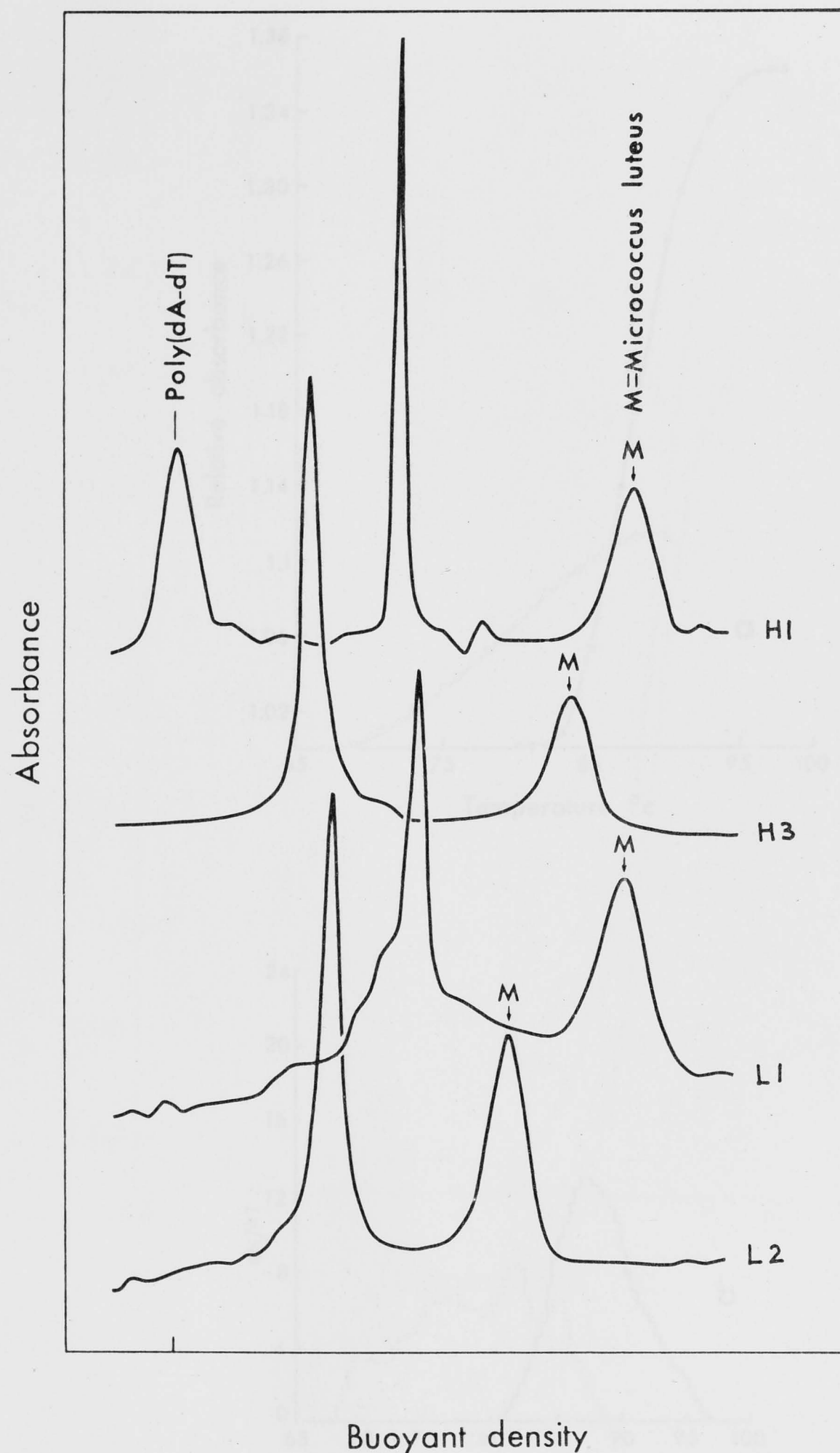


Fig. 5

UV absorbance profiles of reassociated (C_0t about 4) satellite DNAs of *H. vulgare* in analytical CsCl gradients (neutral). M = marker ($\rho = 1.731$ g/cm³ for *Micrococcus luteus* and 1.680 g/cm³ for poly(dA-dT)). Densities are listed in Table II.

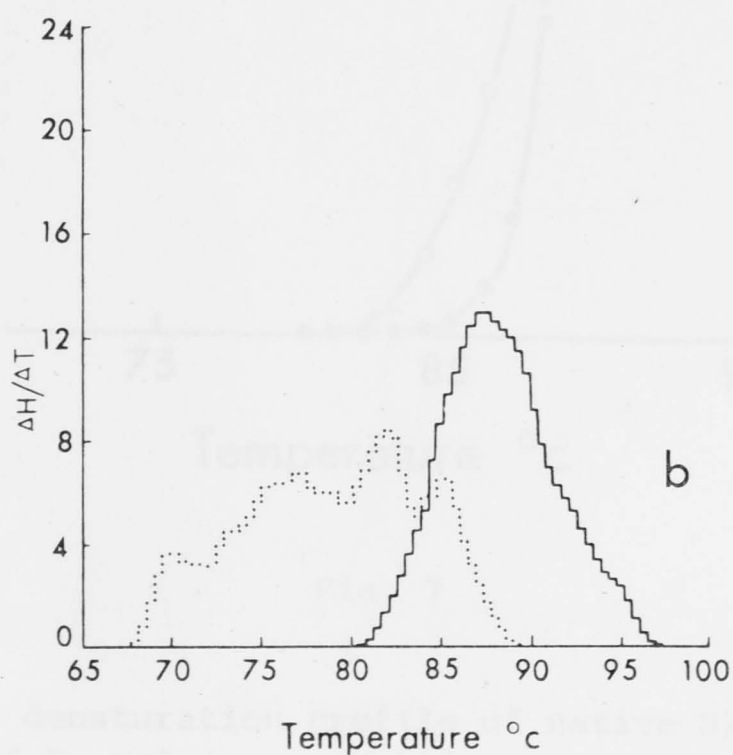
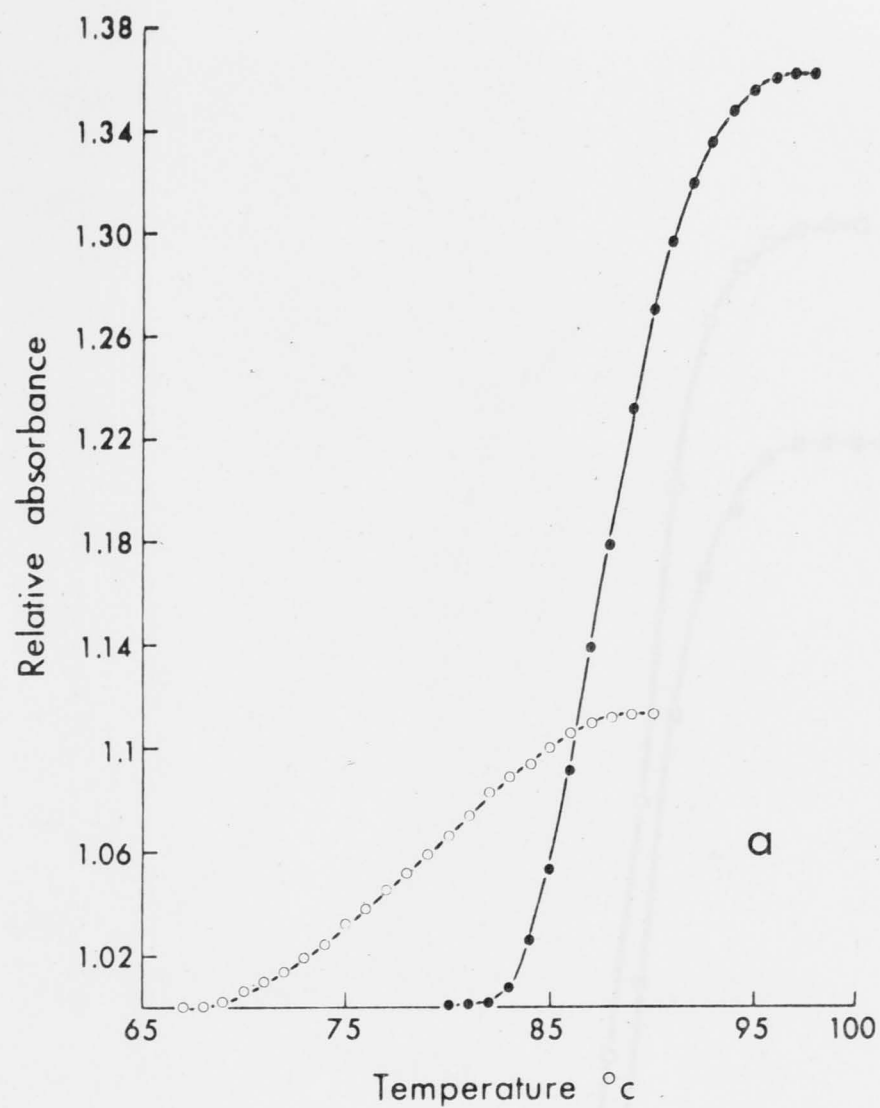


Fig. 6

- a. Thermal denaturation profiles of native (●—●) and reassociated (○—○) H1 satellite of *H. vulgare*.
- b. Differential analysis of Fig. 6a; native DNA (solid line), reassociated DNA (dotted line).

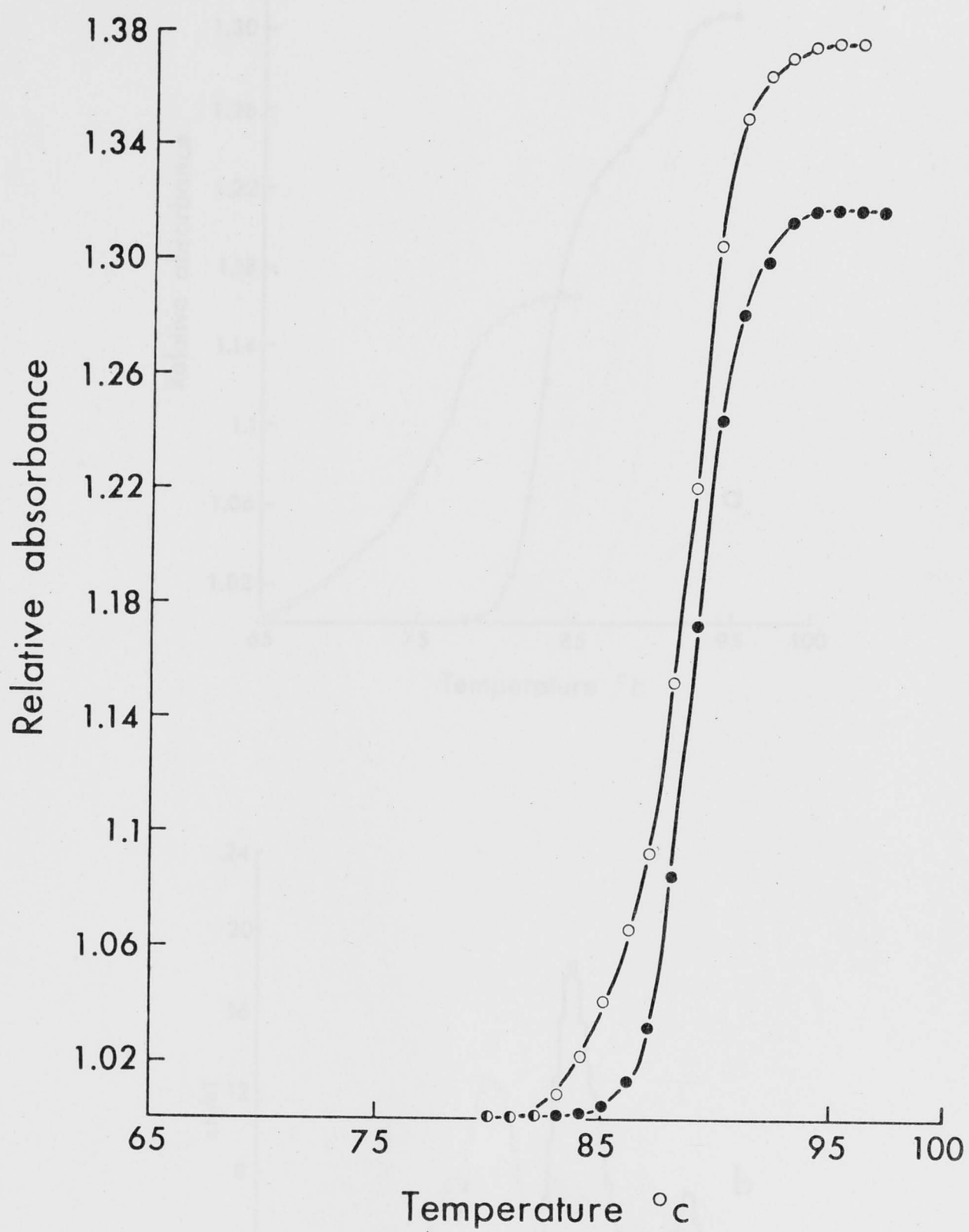


Fig. 7

Thermal denaturation profile of native H2 satellite
 (●—●) DNA of *H. vulgare*. Melting temperature of *E. coli*
 DNA (○—○) was taken as the standard.

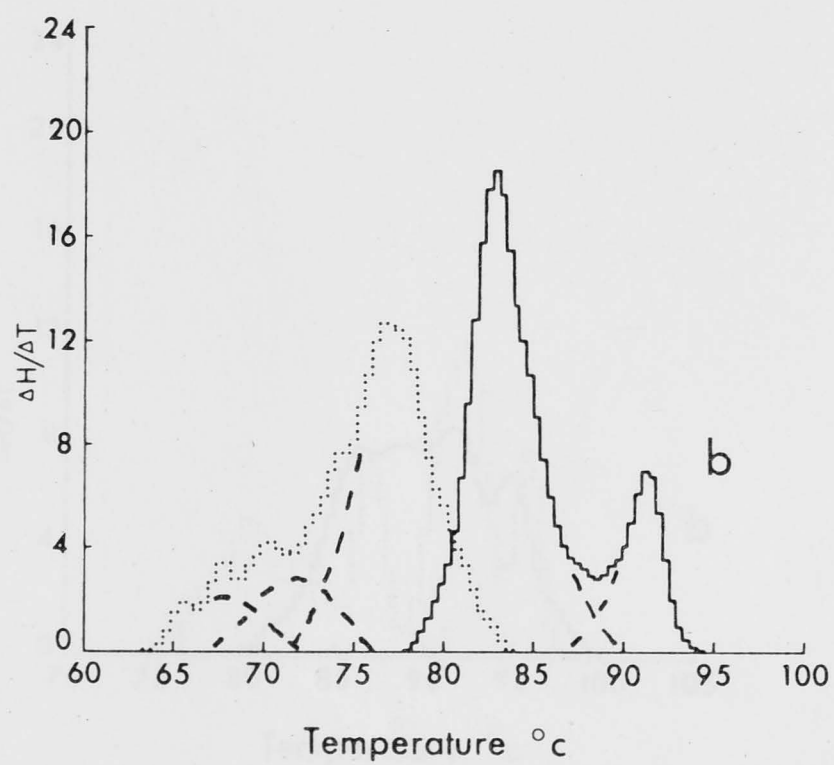
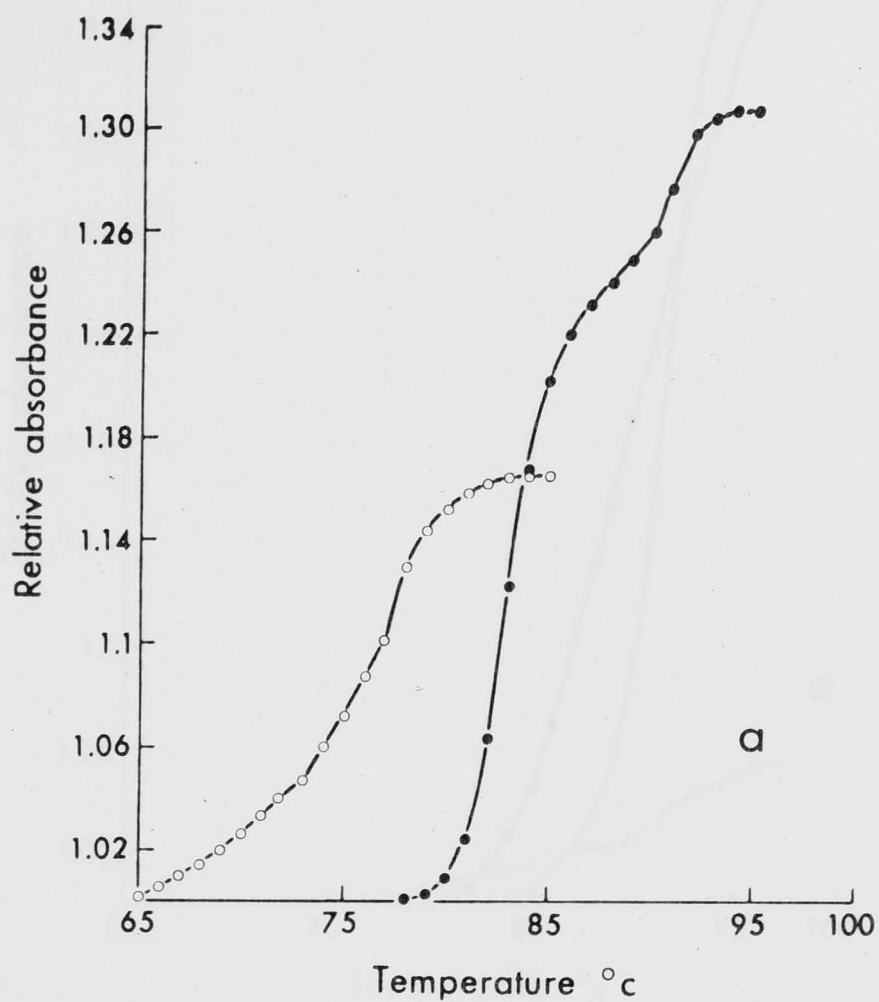


Fig. 8

- a. Thermal denaturation profiles of native (●—●) and reassociated (o—o) H3 satellite of *H. vulgare*.
- b. Differential analysis of Fig. 8a; native DNA (solid line), reassociated DNA (dotted line).

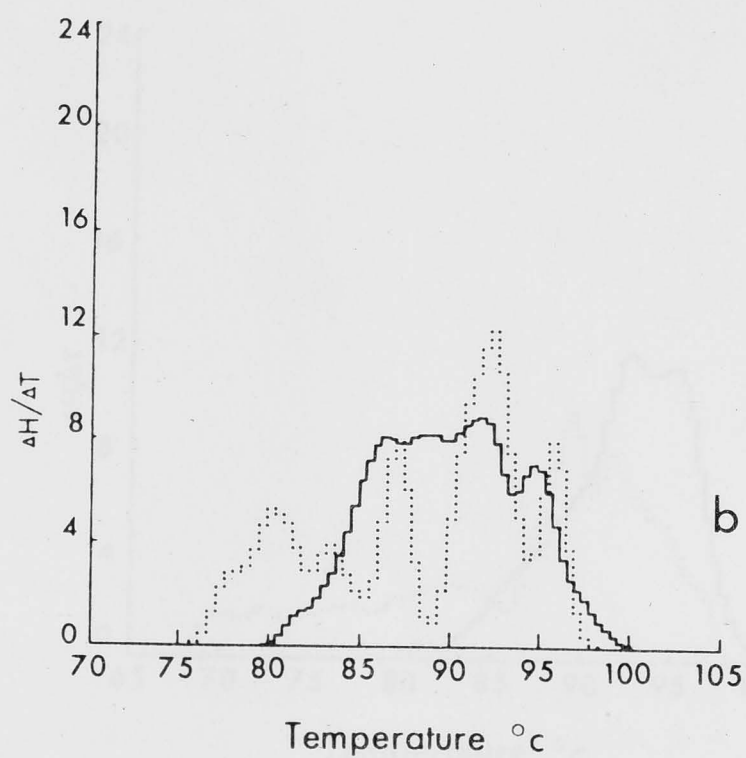
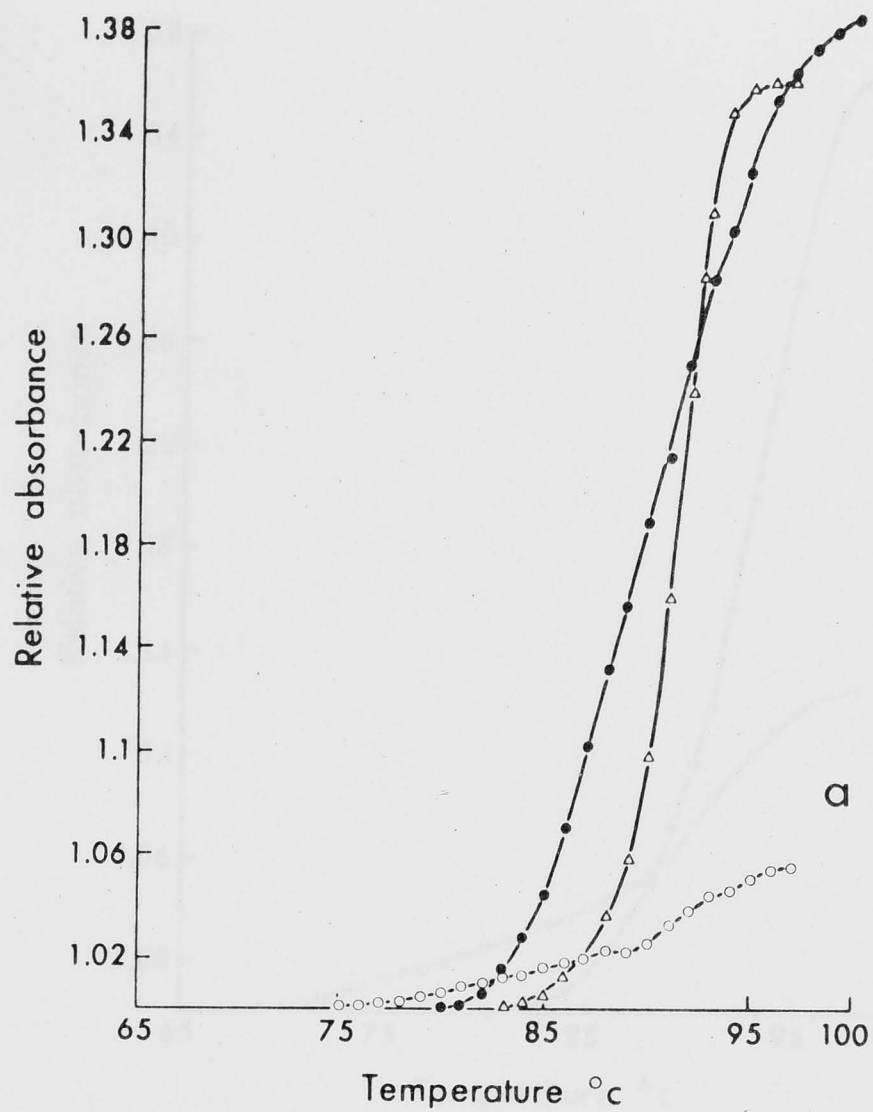


Fig. 9.

- a. Thermal denaturation profiles of native (●—●) and reassociated (o—o) L1 satellite of *H. vulgare* and of *E. coli* DNA (Δ—Δ).
- b. Differential analysis of Fig. 9a (*E. coli* not included), native DNA (solid line), reassociated DNA (dotted line).

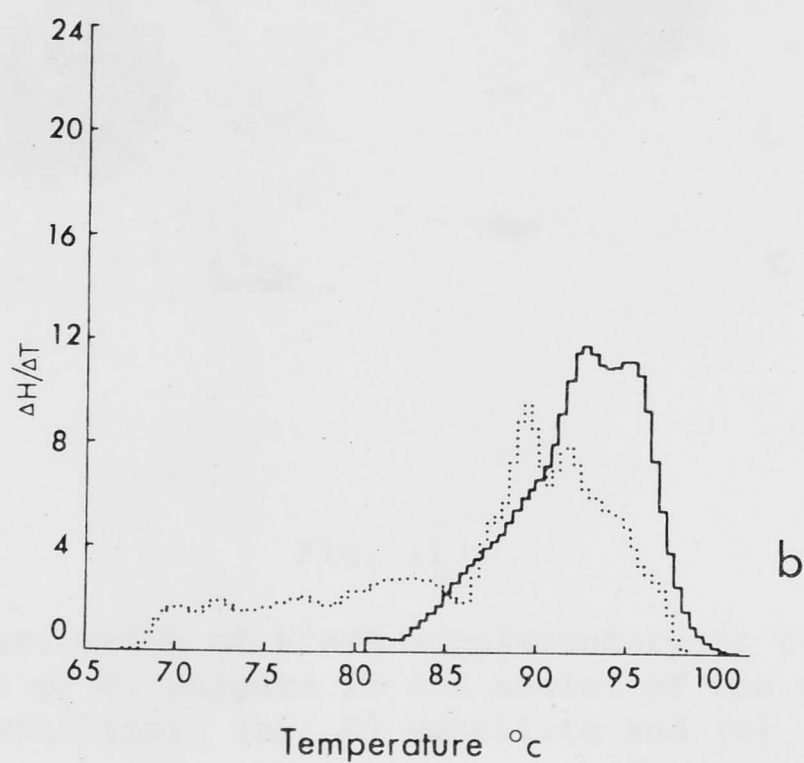
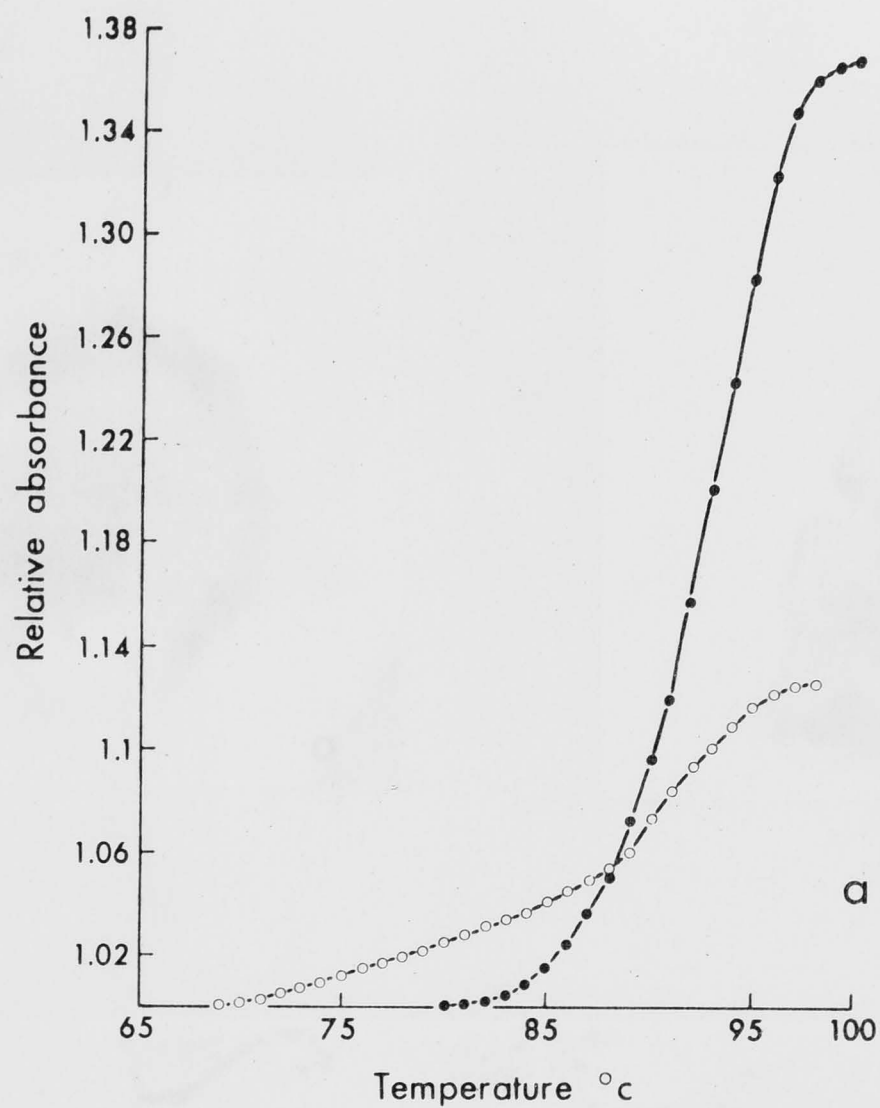


Fig. 10

- a. Thermal denaturation profiles of native (●—●) and reassociated (○—○) L2 satellite of *H. vulgare*.
- b. Differential analysis of Fig. 10a; native DNA (solid line), reassociated DNA (dotted line).

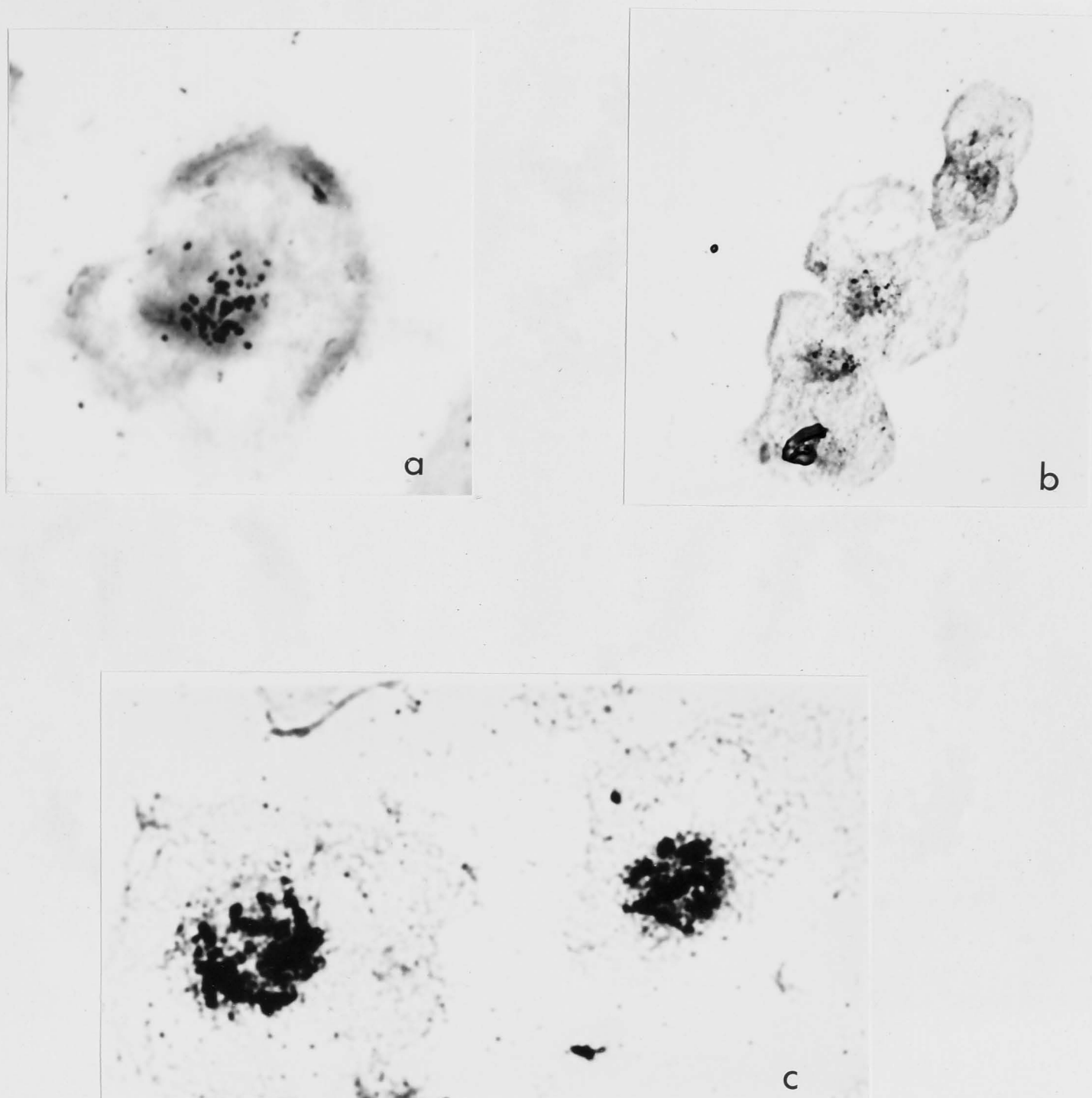


Fig. 11

Distribution of H^3 RNA complementary to three heavy satellites of *H. vulgare* in the nuclei of the same species; (a) H1 satellite; (b) H2 satellite and (c) H3 satellite.

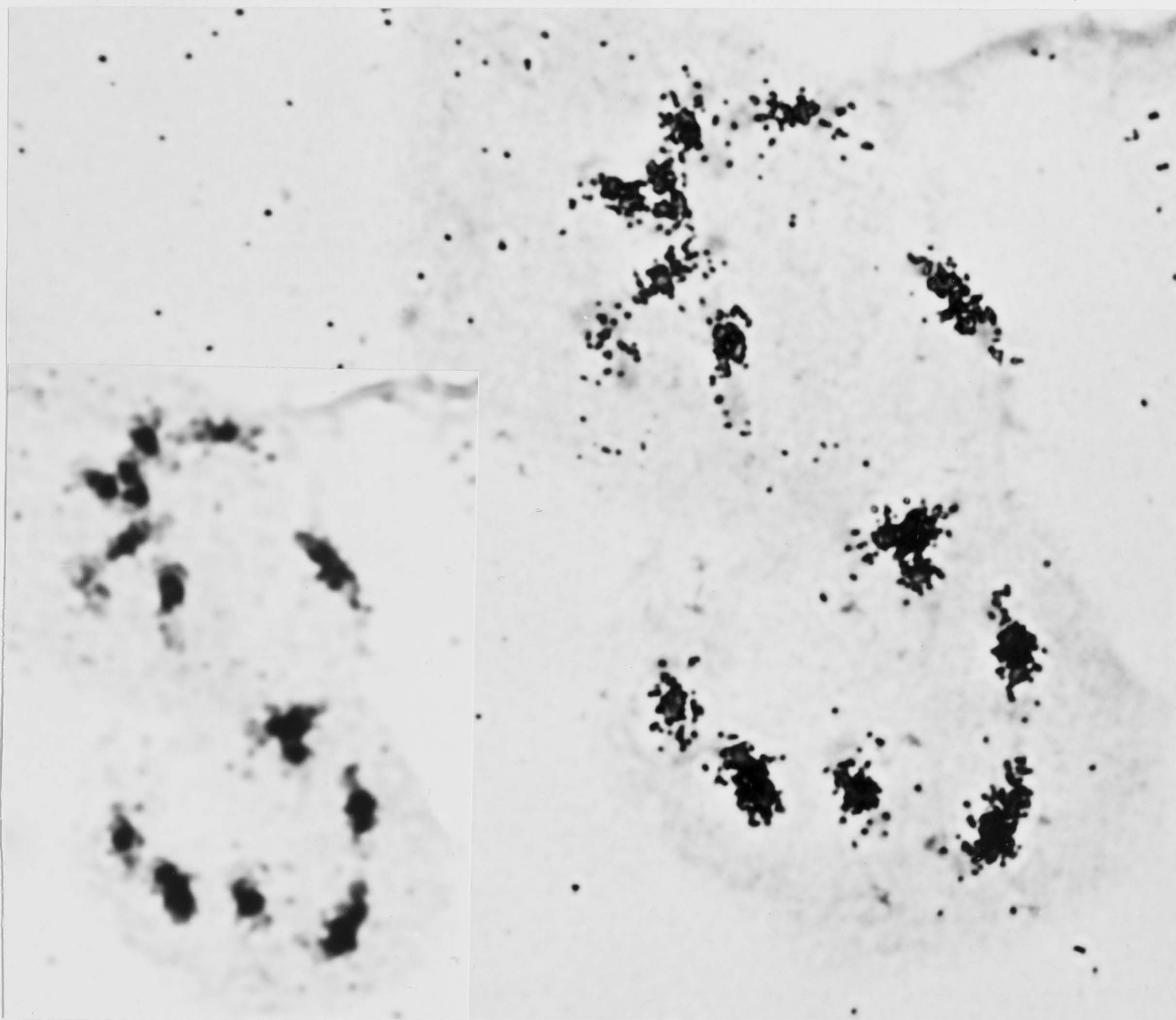


Fig. 12

Distribution of H3 satellite sequences of *H. vulgare* on mitotic chromosomes of the same species ($2n + 1^4 = 15$). H^3C -RNA (7×10^4 cpm/ μ l) was used for *in situ* hybridization.

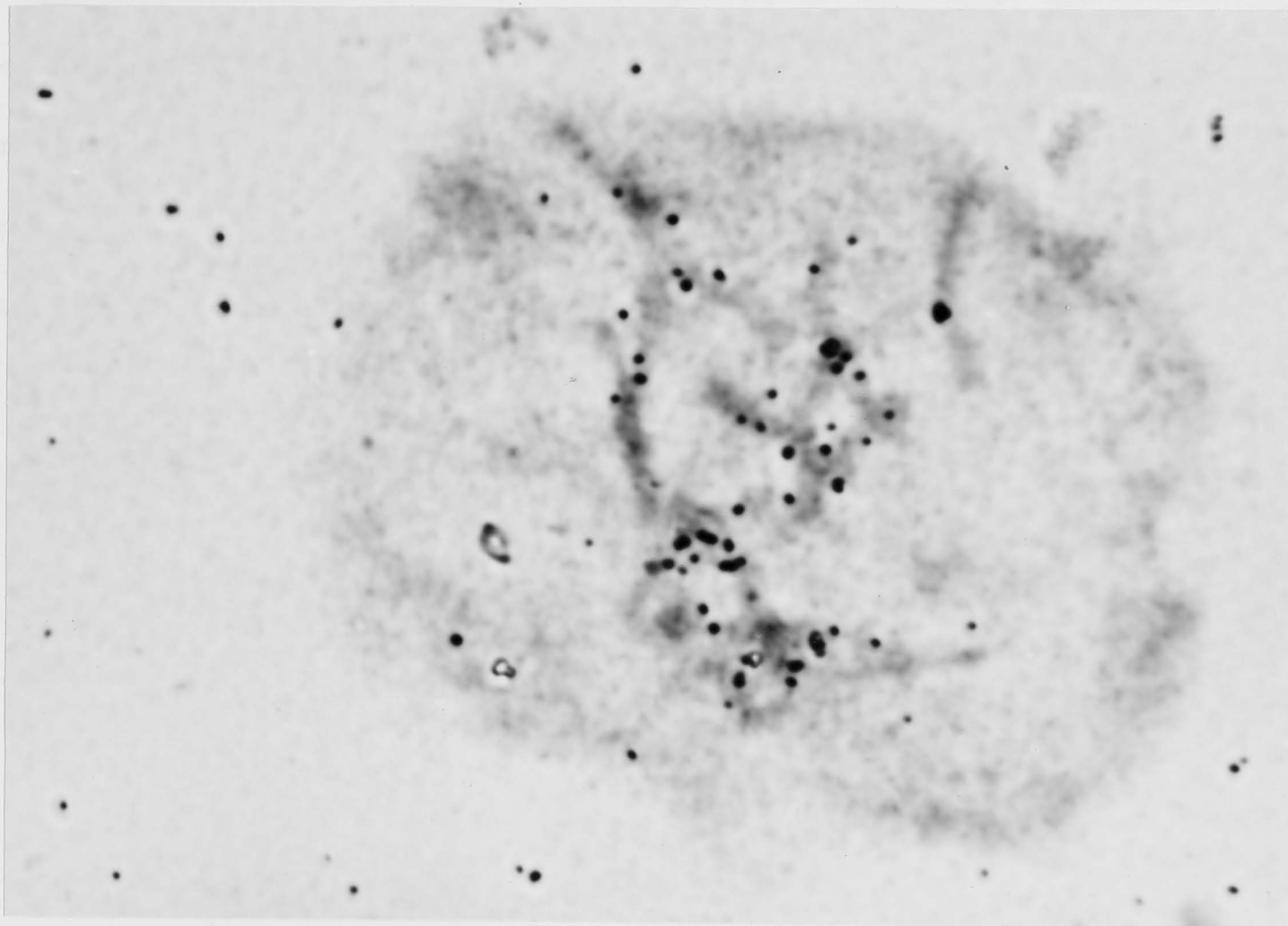


Fig. 13

Distribution of H2 satellite sequences of *H. vulgare* on mitotic chromosomes of the same species. H^3C -RNA (1×10^4 cpm/ μ l) was used for *in situ* hybridization.

(b) *H. arizonicum* satellites

Buoyant density analysis

One heavy and two light satellites were purified by repeated preparative centrifugation in $\text{Ag}^+ - \text{Cs}_2\text{SO}_4$ (Fig. 14). Buoyant densities of the satellites in CsCl are listed in Table IV, and the UV profiles are presented in Fig. 15. One heavy (H1) and one light satellite (L1) each produces rather a broad peak in CsCl; the other light satellite (L2) splits in two.

TABLE IV

Buoyant densities of satellites in CsCl

	Satellite *	Number of component(s) in CsCl	Density (g/cm ³)	% G + C
<i>H. arizonicum</i>	H1	1	1.700	41.06
	L1	1	1.701	42.35
	L2	1	(i) 1.680	21.03
			(ii) 1.661	1.60

* H refers to satellites on the heavy, and L refers to satellites on the light sides of main band DNA.

+ Calculated from buoyant density according to Mandel et al. (1968).

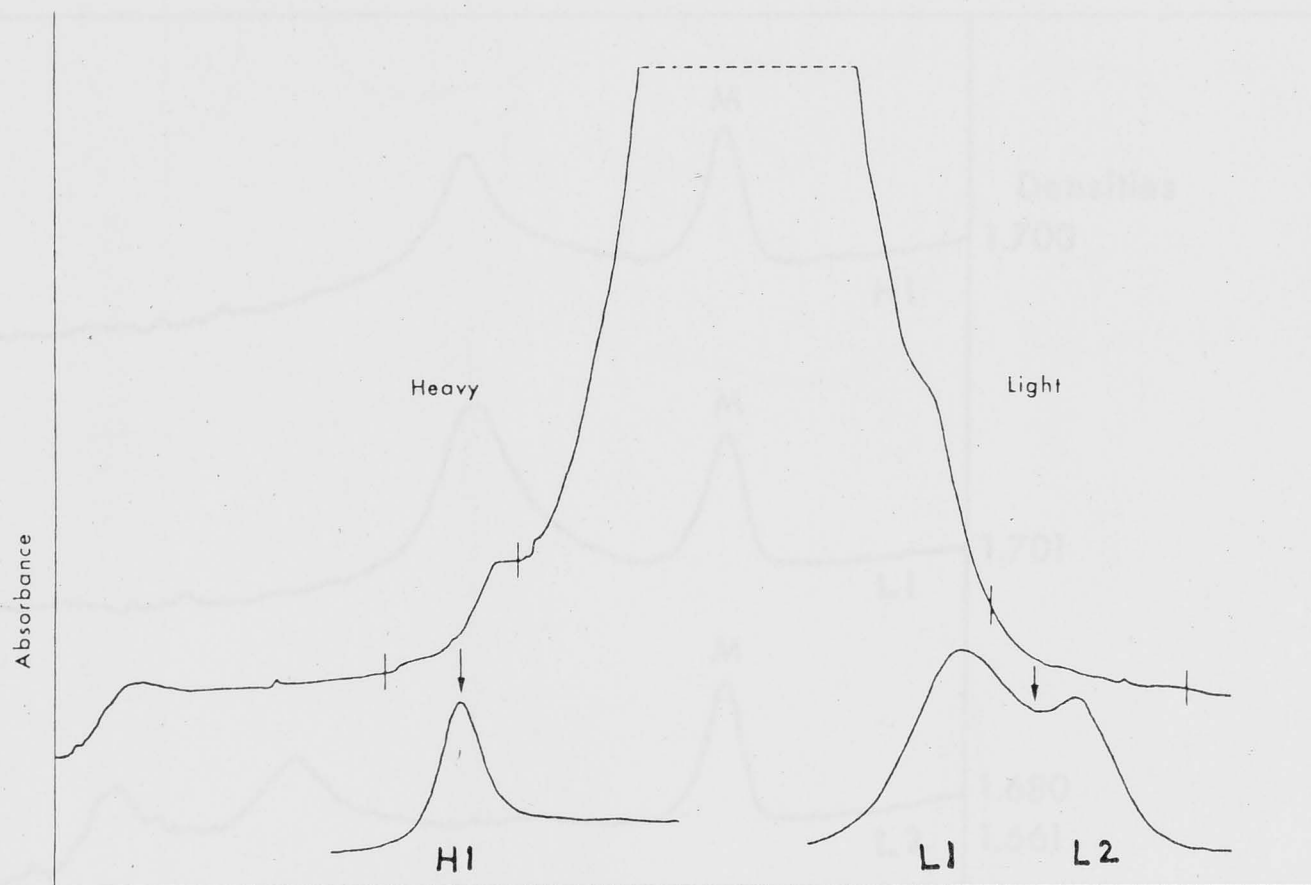


Fig. 14

UV absorbance profiles of *H. arizonicum* DNA after preparative ultracentrifugation in Ag^+ - Cs_2SO_4 gradients. Upper figure represents the first preparative step. Fractions selected for each satellite are shown by vertical lines on the figures. The preparative step was repeated using pooled fractions for each satellite from several gradients; their absorbance profiles are shown in the lower figures. Originally the lower figures were of the same scale as the upper one, but are shown in reduced size. A third preparative step was necessary to separate L1 and L2.

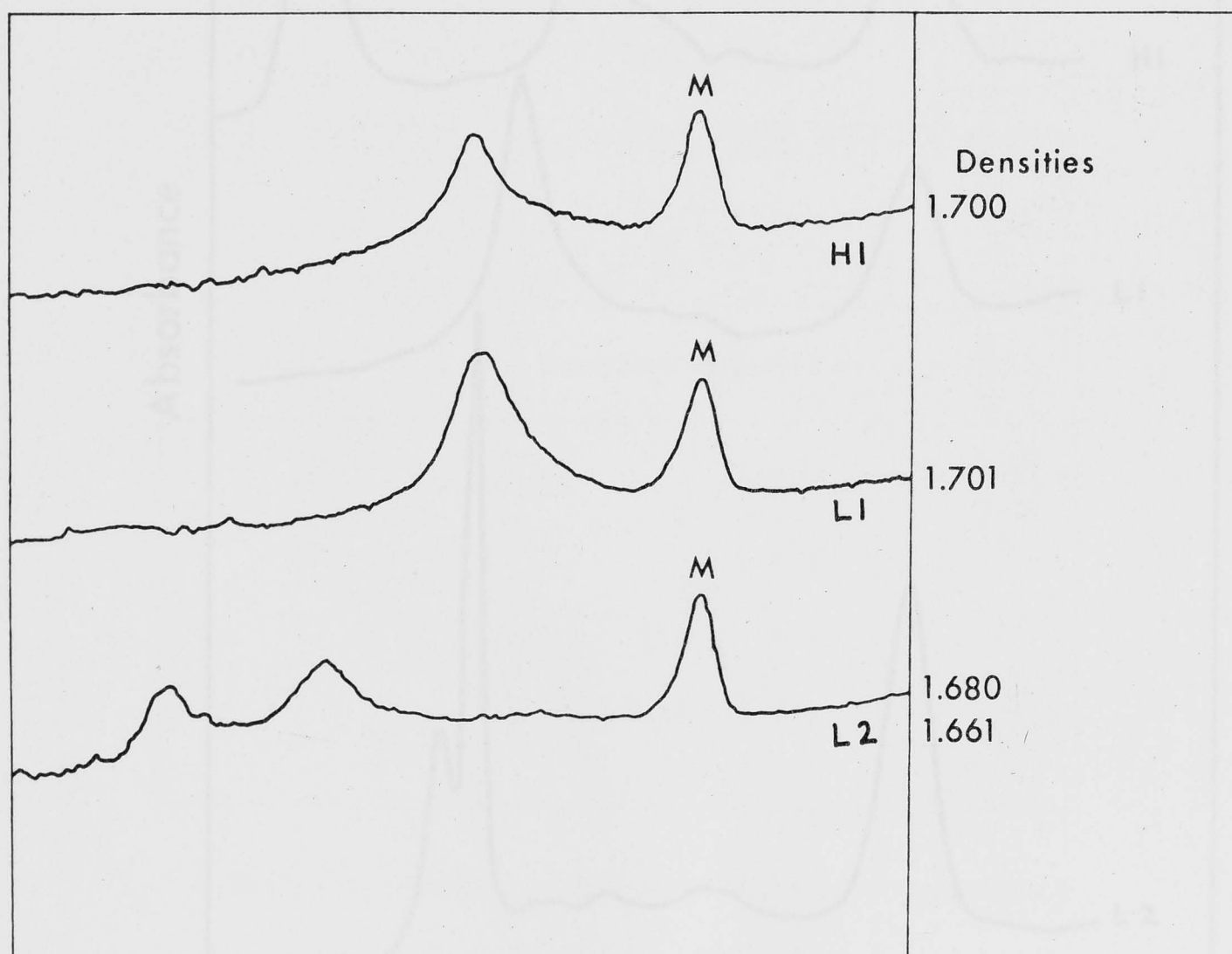


Fig. 15

UV absorbance profiles of *H. arizonicum* satellite in analytical CsCl gradients (neutral). Densities of the components are shown on the right. M refers to marker DNA (*Micrococcus luteus*, $\rho = 1.731$ g/cm³).

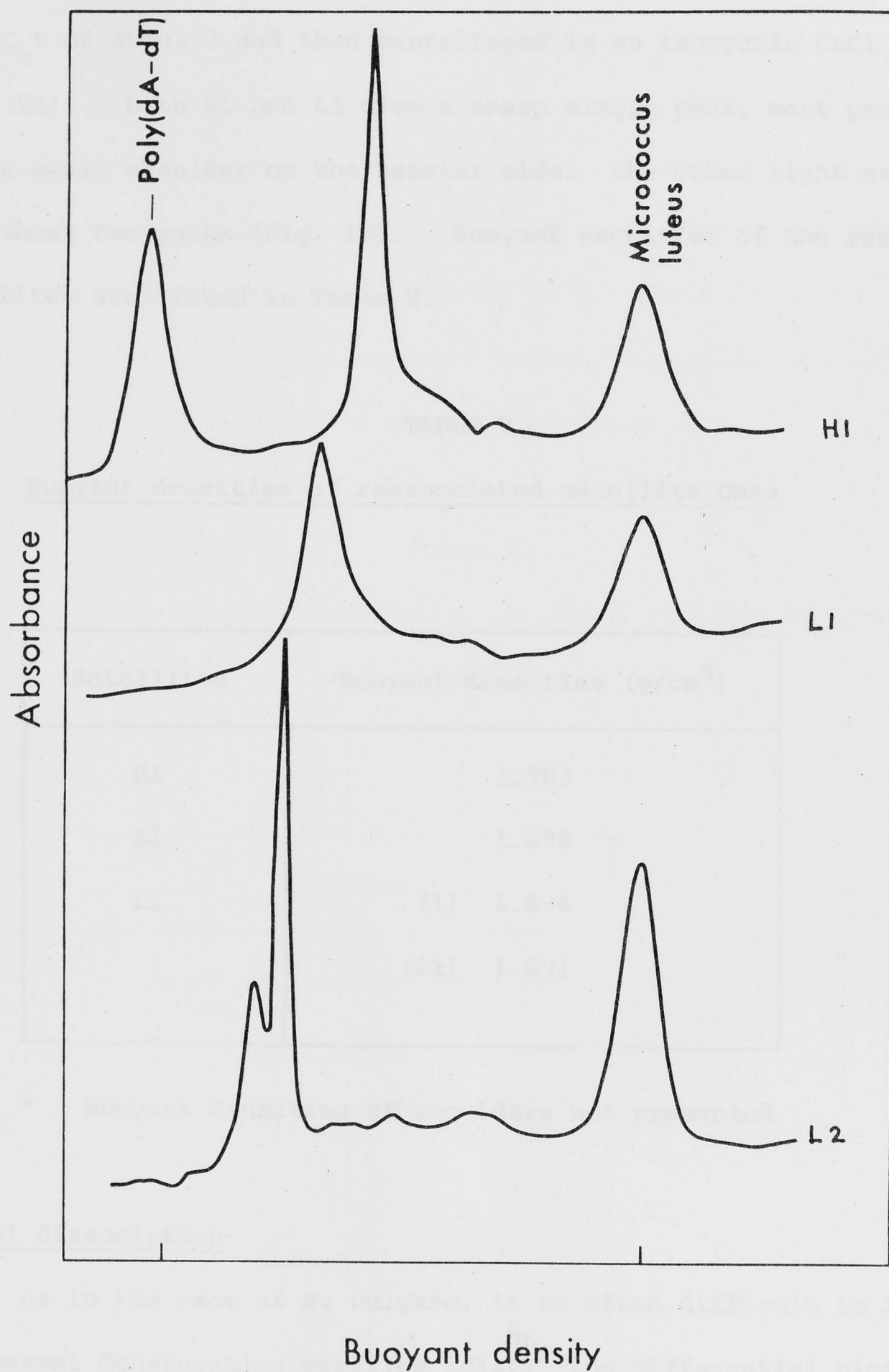


Fig. 16

UV absorbance profiles of reassociated (C_0t about 3) satellite DNAs of *H. arizonicum* in analytical CsCl gradients (neutral). *Micrococcus luteus* DNA ($\rho = 1.731 \text{ g/cm}^3$) or poly(dA-dT) ($\rho = 1.680 \text{ g/cm}^3$) or both were used as reference marker(s).

After denaturation, each satellite DNA was allowed to reassociate to a C_0t of about 3 and then centrifuged in an isopycnic CsCl gradient (Fig. 16). Both H1 and L1 give a sharp single peak, most probably with a small shoulder on the heavier side; the other light satellite (L2) shows two peaks (Fig. 16). Buoyant densities of the reassociated satellites are listed in Table V.

TABLE V

Buoyant densities of reassociated satellite DNAs

Satellites	Buoyant densities (g/cm ³)
H1	1.703
L1	1.698
L2	(i) 1.694
	(ii) 1.691

* Buoyant densities of shoulders not presented

Thermal dissociation

As in the case of *H. vulgare*, it is often difficult to interpret the thermal denaturation profiles fully; the differential plot, however, reveals the full complexity of denaturation. The melting temperatures of the satellites are presented in Table VI. The same considerations which were followed for *H. vulgare* satellites, were used when determining T_m 's from the differential plots. The native heavy satellite (H1) melts with a seemingly biphasic transition, indicating at least two components (Fig. 17a) but the differential plot strongly suggests the presence of three components with T_m 's at 82.5 C, 87 C and 91 C respectively (Fig. 17b). The seemingly simple denaturation

profile of the reassociated H1 turns out to be complex on differential analysis and has possibly four components, the principal one with a Tm of 75 C. Thermal denaturation of L1 satellites also shows a multi-component pattern (Fig. 18). By contrast, the native L2 DNA gives a 'nearly perfect' thermal transition profile (Tm = 86 C) with the possibility of a minor component at Tm 95 C. Reassociated L2 is, however, complex, with probably four components having Tm's of about 67.5 C, 73 C, 77.5 C and 82 C respectively (Fig. 19).

TABLE VI
Melting temperatures (Tm's) of native and reassociated
satellites

Satellite	Native		Reassociated	
	Tm of native [*] DNA (c)	Thermal com- ponents	Tm of reasso- [*] ciated DNA (c)	Thermal components
H1	82.5 [†] , 87, 91	3	67, 71.5, 75 [†] , 82	4
L1	86.5 [†] , 92, 97.5	3	70, 75, 81.5 [†] , 87	4
L2	86 , 95	2	67.5, 73, 77.5 [†] , 82 [†]	4

* Determined from the differential plot after reconstruction from the contributing components.

† Major component.

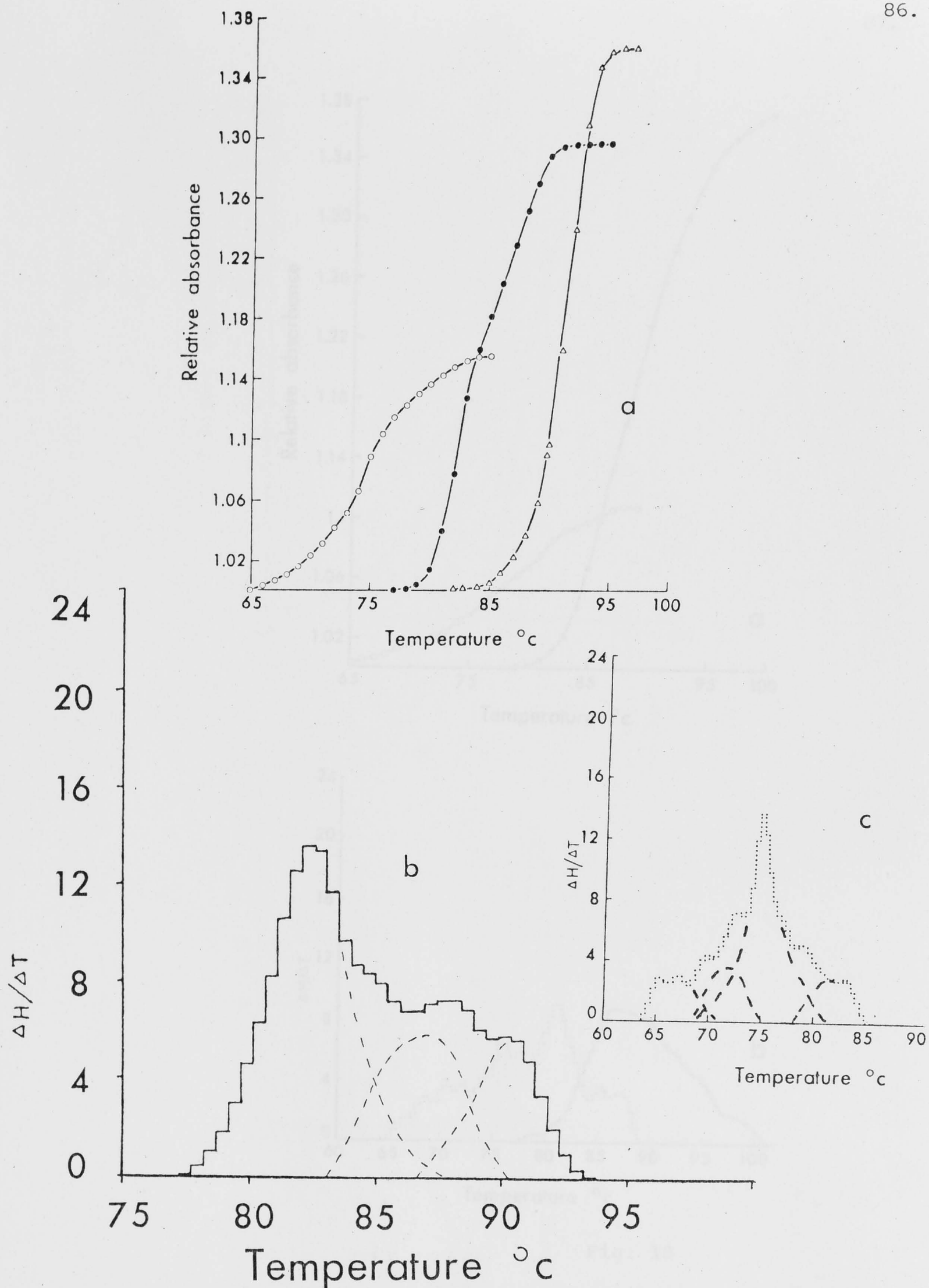


Fig. 17

- a. Thermal denaturation profiles of native (●—●) and reassociated (○—○) H1 satellite DNA of *H. arizonicum* and of native *E. coli* DNA (Δ — Δ).
- b. Differential analysis of denaturation data for native H1 DNA (Fig. 17a). Contributing components are shown with dotted lines under the curve.
- c. Same as (b) for reassociated H1 DNA.

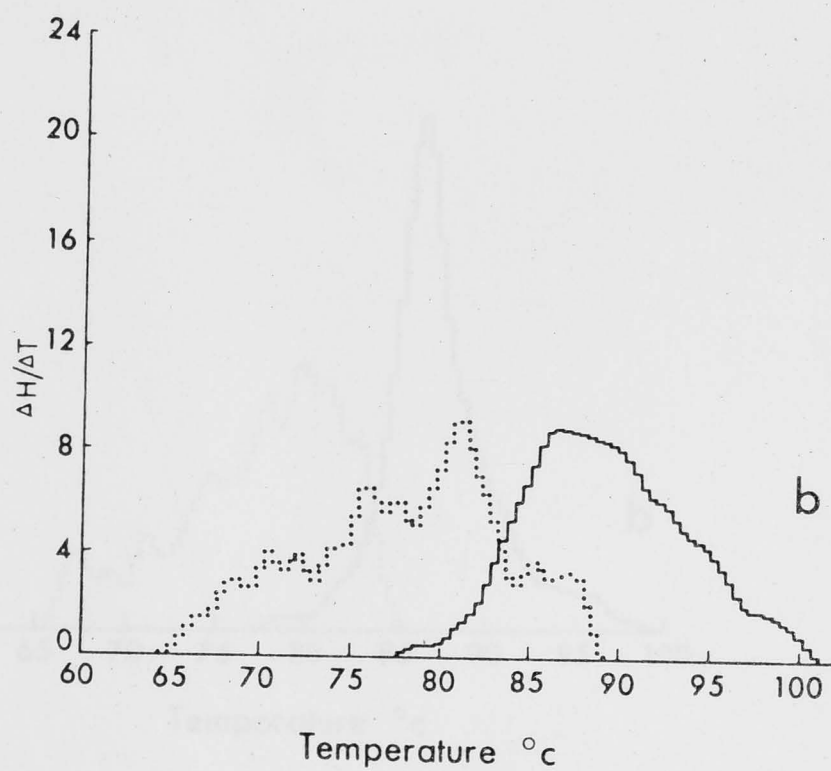
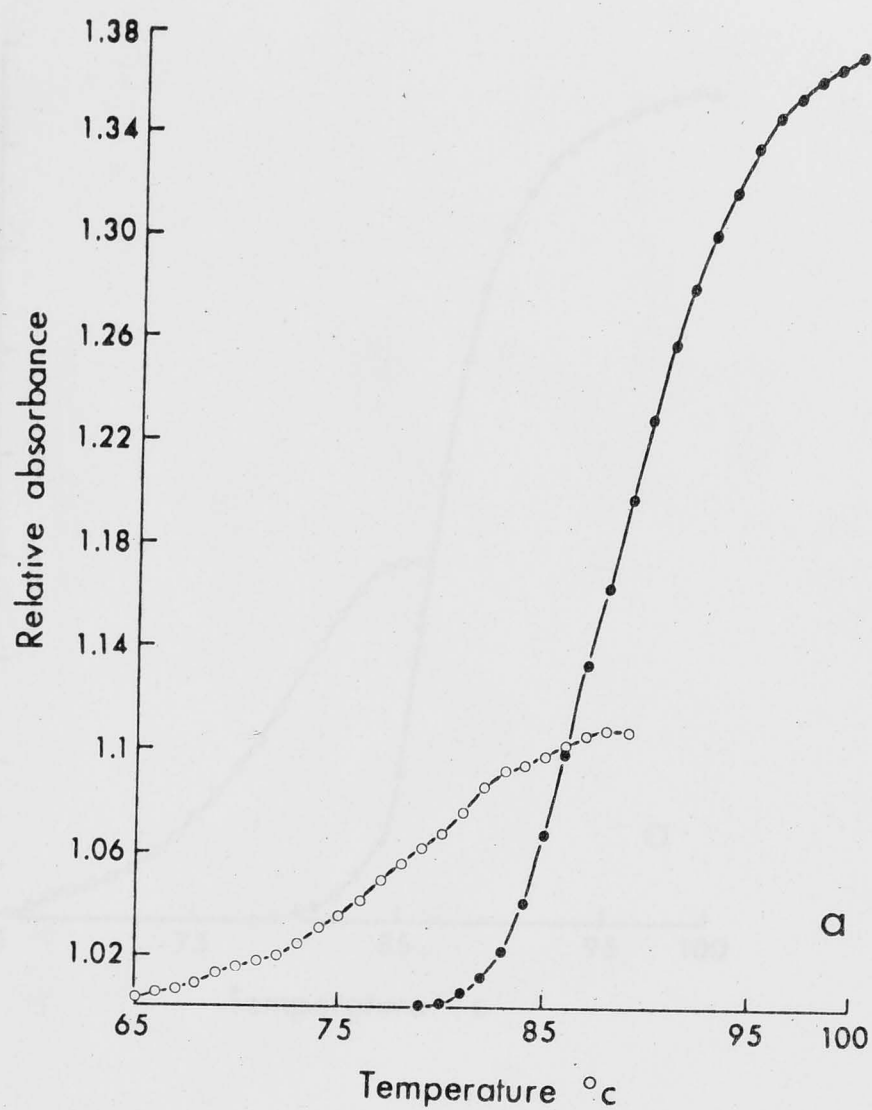


Fig. 18

- a. Thermal denaturation profiles of native (●—●) and reassociated (○—○) L1 satellite DNA of *H. arizonicum*.
- b. Differential analysis of Fig. 18a; native DNA (solid line), reassociated DNA (dotted line).

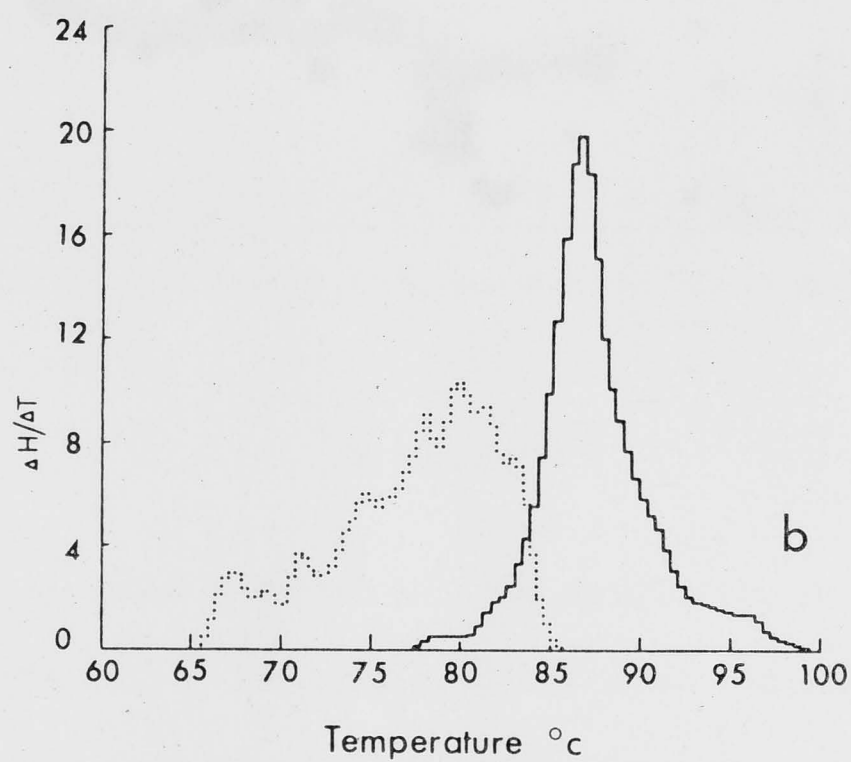
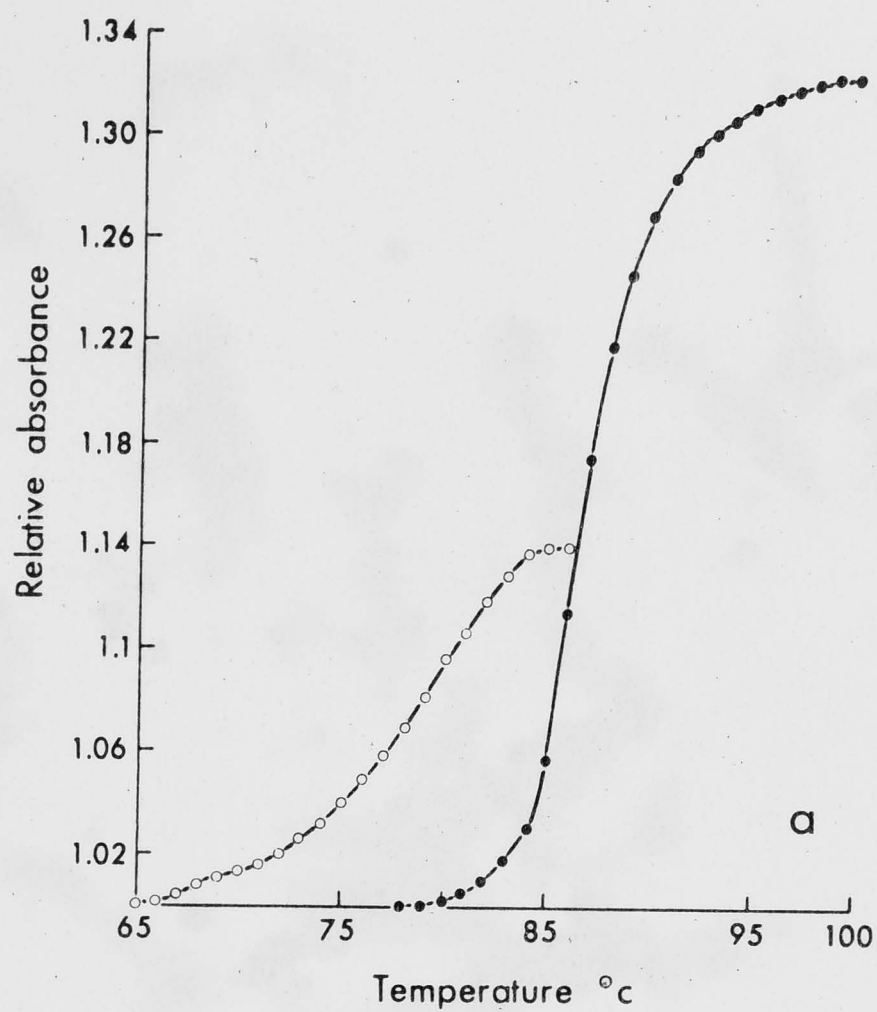


Fig. 19

- a. Thermal denaturation profiles of native (●—●) and reassociated (○—○) L2 satellite DNA of *H. arizonicum*.
- b. Differential analysis of Fig. 19a; native DNA (solid line), reassociated DNA (dotted line).

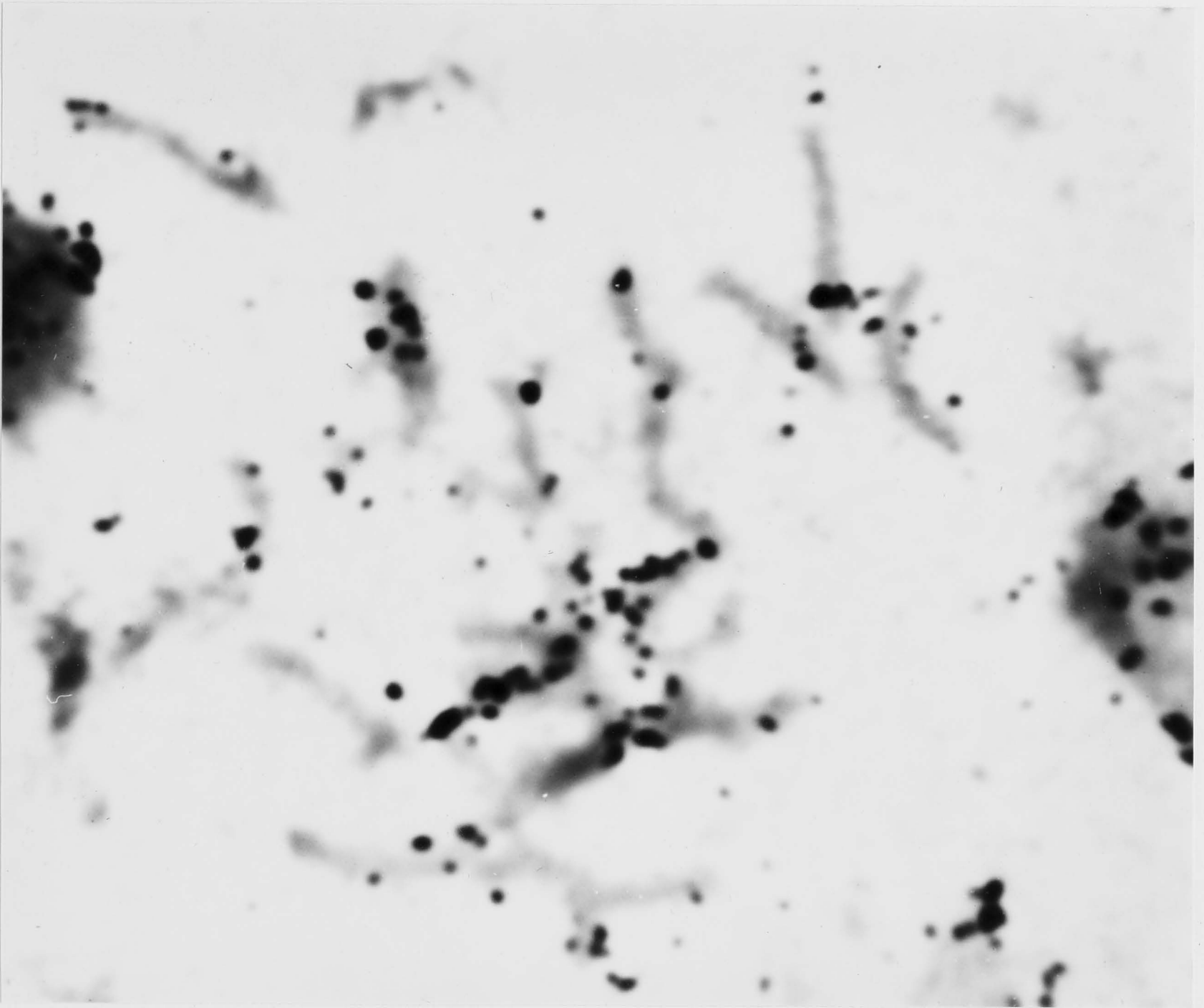


Fig. 20

Distribution of H1 sequences of *H. arizonicum* in the nuclei and mitotic chromosomes of *H. arizonicum* ($3n = 21$). H^3C -RNA (6×10^4 cpm/ μ l) was used for *in situ* hybridization.

In situ hybridization

Radioactive complementary RNA (H^3 -cRNA) synthesized from each of the satellites was hybridized with chromosomes of *H. arizonicum*. For convenience of chromosome spread polyhaploid plants ($n = 3x = 21$) were used (kindly supplied by Dr N. C. Subrahmanyam). These were obtained following differential chromosome elimination in *H. arizonicum* x *H. vulgare* (Subrahmanyam, personal communication). The same *in situ* hybridization procedure was used throughout, but again only the heavy satellite hybridized well with the chromosomes (Fig. 20). Light satellites, on the other hand, do not show any hybridization at all, even after two months exposure. As in the case of the light satellites of *H. vulgare* no attempts were made to optimize the steps involved in the hybridization procedure.

While it was not possible to carry out a complete karyotypic analyses, it does appear that at least some of the chromosomes have little or none of the heavy satellite. In those which are obviously labelled, their label is not always found in presumptive centromeric regions.

DISCUSSION

The physical properties of all the satellites described here are summarised in Table VII. Density gradient analysis reveals that the light satellites are more complex in their base composition than the heavy ones. Except in the case of the H2 satellite of *H. vulgare*, where no reassociation was attempted, each native and reassociated heavy satellite forms a single band in a CsCl gradient and the buoyant density of the latter is not much different from that of the native DNA (Table VII), indicating very little sequence mismatch. Each satellite was purified as a single component in a preparative Ag^+ - Cs_2SO_4 gradient, yet three of the four light satellites reveal the presence

of more than one component upon analytical ultracentrifugation in CsCl gradients (Table VII). A similar observation has been reported for the light satellite of rye (Appels et al., 1978). The L1 of *H. arizonicum* is different from other light satellites in showing only one peak in a CsCl density gradient profile.

The complicated nature of the three light satellites mentioned previously is also apparent from the buoyant densities of reassociated satellite DNAs which are much heavier than those of their respective native DNAs except that the density of reassociated L1 DNA of *H. arizonicum* is slightly lower (0.003) than that of its native DNA. This latter observation is very unusual in the sense that even if one obtains perfect reassociation the density should be the same as that of the native form, not lower.

At alkaline pH (9.2 was used in this study) Ag^+ binds preferentially to the A + T-rich fraction of calf DNA (Filipskiet et al., 1973; Macaya, 1976). If this is true for all eukaryotic DNA then heavy satellites, as a whole, must be different from the light satellites in their base composition. This is because these two types of satellites band in quite different densities in a $\text{Ag}^+ - \text{Cs}_2\text{SO}_4$ gradient and are separated by a colossal main band. The buoyant densities of three of the four heavy satellites are very close to that of the main band DNA, consequently % G + C contents determined from buoyant densities are also very close. On this criterion only the H2 satellite of *H. vulgare* is G + C-rich (69% compared to about 41% for other heavy satellites). On the other hand, with the exception of the L1 satellite of *H. arizonicum*, light satellites have density components (in CsCl) which appear to be A + T-rich. Moreover, the % G + C content obtained for each satellite by using two established procedures (buoyant density and T_m) is markedly different. The anomalous nature

of satellite DNAs could be explained by assuming that "the normal relationships between base composition, buoyant density and temperature of denaturation do not necessarily apply to satellite DNA" (Corneo *et al.*, 1968). Ranjekar *et al.* (1978) who made similar observations in barley and wheat, suggested that satellite DNA may be conformationally different from main band DNA.

The purified 1.701 g/cm^3 component of the C_{ot} 0.02 fraction of rye DNA is known to produce a hypersharp peak in CsCl and electron microscopic evidence suggests that this is due to aggregate formation, a characteristic of renatured DNA where the repeating sequence is shorter than the fragment length (Appels *et al.*, 1978). If this is a general phenomenon for highly repeated plant DNA, this would explain the observed hypersharp nature of reassociated satellite DNAs of barley.

The L2 satellite of *H. vulgare* shows the presence of four components in CsCl, but after renaturation it bands as a single sharp peak. This could only be explained by assuming that the base compositions of the individual components are similar enough to allow cross hybridization. Arguing from the same grounds, it can be concluded that two components of the L1 satellite of *H. vulgare* have similar base sequences. The L2 DNA of *H. arizonicum* consists of two density classes and unlike both the L1 and the L2 of *H. vulgare*, these classes retain their identity after renaturation.

Earlier studies of thermal denaturation of satellites utilized plant species where only one satellite is present. In *H. vulgare* only one of the two satellites (Ranjekar *et al.*, 1976) was studied by Ranjekar *et al.*, (1978). When thermal denaturation profiles of satellite DNAs from different species are compared, some of them are monophasic (Capesius, 1976; Timmis and Ingle, 1977; Ranjekar *et al.* 1978) while others are biphasic (Bendich and Anderson, 1974; Sinclair *et al.*, 1975; Chilton, 1975; Deumling and Nagl, 1978). The satellites

of *Hordeum* species also fall into these two classes. The use of differential analysis in the present work reveals that the situation is often more complicated than the thermal transition profile suggests.

The physical properties of *H. vulgare* H3 satellite and of *H. arizonicum* H1 satellite are very similar (Table VII). Both satellites are similar in respect of the buoyant densities of native and reassociated DNAs, the biphasic melting profiles, the T_m of two thermal components and the T_m of the major reassociated component. The only significant difference appears to be the occurrence of an additional thermal component ($T_m = 87^\circ\text{C}$) in the H1 of *H. arizonicum*. It is reasonable, therefore, to suggest that the base composition of the two similar thermal components are also similar in these two species.

There is other evidence which suggests that each species has its own characteristic sequences. In *H. vulgare*, for example, in addition to H3, there are two more heavy satellites, the last two being undetectable in *H. arizonicum*. Similarly there are differences in the components of the light satellites. While it is realised that sequencing data is necessary to prove conservation of sequences among different species of *Hordeum*, the question of conservation of satellites will be discussed further in the next Chapter which describes the *in situ* hybridization using H^3 c-RNA of these two satellites and chromosomes of other *Hordeum* species.

The H3 satellite most probably corresponds to the 'satellite I' of *H. vulgare* described by Ranjekar *et al.* (1978). The difference in buoyant density reported in the two studies is consistent with the small difference observed for main band DNA also. The main point is that the density of H3 satellite is very close to that of the main band DNA which is exactly what Ranjekar *et al.* (1978) reported. Other similar properties include biphasic thermal transition, T_m of both components and T_m of reassociated satellites (Table VII).

TABLE VII

Physical characteristics of the satellite DNAs of *H. vulgare* and *H. arizonicum*

1 Species	2 Satellite	3 Component(s) in CsCl	4 Buoyant density (native DNA) g/cm ³	5 % G + C content (from ρ) [†]	6 Buoyant density (reassociated DNA) g/cm ³	7 Difference in 6 and 4 g/cm ³	8 T _m native DNA °C	9 Component(s) in differential analysis	10 % G + C content of thermal components ‡	11 T _m (reassociated DNA) °C	In situ hybridization
<i>H. vulgare</i>	Heavy										
	H1	1	1.700	41.6	1.700	0	87 [†] , 93	2	43, 58	71, 75.5 [†] , 80.5 [†] , 85	✓
	H2	1	1.727	69.2	not done		91	1	53	not done	✓✓
	H3	1	1.699	40.1	1.703	+ 0.004	83 [†] , 91.5 (80%) (20%)	2	34, 54	67, 71, 77 [†]	✓✓✓
	I(a)	1(a)	1.702(a)	42.8(a)			82.5 91(a) (80%) (20%)	2(a)	32.3, 52.9(a)	75.5(a)	not reported
<i>H. arizonicum</i>	H1	1	1.700	41.6	1.703	+ 0.003	82.5 [†] , 87, 91	3	33, 43, 53	67, 71.5, 75 [†] , 82	✓✓✓
<i>H. vulgare</i>	Light										
	L1	2(a)	1.697 1.688	38.2 28.6	1.708 [*]	**	86 [†] , 90, 95.5, 94.8	3	41, 51, 64	80, 87, 92.5, 95.5 [†]	none
	L2	4(a) (b) (c) (d)	1.713 1.706 1.696 1.685	54.4 47.6 37.5 25.7	1.712	**	86.5, 91, 95 [†]	3	42, 53, 63	87, 91, 95 [†]	none
	L1	1	1.701	42.4	1.698	- 0.003	86.5 [†] , 92, 97	3	42, 55, 69	70, 75, 81.5 [†] , 87	none none
	L2	2(a) (b)	1.680 1.661	21.3 1.6	1.694 1.691	+ 0.014 0.030	86 [†] , 95	2	41, 63	67.5, 73, 77.5 [†] , 82 [†]	none
<i>H. arizonicum</i>	L1	1	1.701	42.4	1.698	- 0.003	86.5 [†] , 92, 97	3	42, 55, 69	70, 75, 81.5 [†] , 87	none none
<i>H. arizonicum</i>	L2	2(a) (b)	1.680 1.661	21.3 1.6	1.694 1.691	+ 0.014 0.030	86 [†] , 95	2	41, 63	67.5, 73, 77.5 [†] , 82 [†]	none

[†] - Determined according to Mandel et al. (1968); [‡] - Calculated according to Mandel and Marmur (1968);
[†] - Major component(s); ** - Difficult to determine; * - Buoyant density of shoulders not shown; ✓ - Degree
of hybridization with the same species; (a) - Data of Ranjekar et al. (1978) for 'satellite I' of *H. vulgare*. See
Discussion for similarity with H3 satellite of this study.

Attempts to hybridize H^3 -RNA complementary to the heavy satellites of both species with chromosomes of the corresponding species were successful. The procedures for cRNA synthesis and *in situ* hybridization were the same for heavy and light satellites, yet no hybridization was observed between H^3 c-RNA of light satellites and chromosomes. No special attempt was made to optimize any of the steps involved in *in situ* hybridization. The simplest explanation for failure of hybridization is that the light satellites of these two species do not contain highly repeated DNA as has been suggested for the light satellite of rye (Appels et al., 1978). Since binding of Ag^+ to DNA is specific at alkaline pH (Filipski et al. 1973), the banding of satellites at the lighter side of the main band indicates that these sequences are compositionally or conformationally different from the rest of the DNA. Composition and conformation may not be mutually exclusive, since the former may influence the latter. A third explanation for the failure of hybridization of the light satellites may be that these sequences are highly interspersed among other non-repeated DNA.

The buoyant densities of component (a) of L1 and (c) of L2, are similar to those reported for plant chloroplast DNA (Wells and Ingle, 1970; Kung, 1977). Component (b) of L2 DNA has a buoyant density which is similar to the density of mitochondrial DNA of plants (Wells and Ingle, 1970). The similarities of the densities may be coincidental, but one can not rule out the possibility that they are contributed by organelle DNA since the source of the DNA was leaf tissue.

In situ hybridization studies using H^3 c-RNA of satellites show the location(s) of the satellite sequences on the chromosomes and give a characteristic pattern to individual chromosomes. In rye, the heterochromatic regions (C-band positive) of the chromosomes are the sites where the highly repeated sequences are mostly located (Appels, 1978). In *Scilla* too, satellite DNA has been reported to be localized at the heterochromatic parts of the chromosomes which are again distal

Timmis et al., 1975).

The C-banding pattern has been published for *H. vulgare* (c.v. York, Noda and Kasha, 1976; c.v. 'Emir', Linde-Laursen, 1976). According to Noda and Kasha (1976), C-bands are mainly located adjacent to centromeres so that the distal regions are devoid of C-bands. In 'Emir' barley telomeres of some chromosomes and secondary constrictions of chromosome 6 and 7 are C-band positive (Linde-Laursen, 1976). Otherwise, in both the studies, the bulk of the heterochromatin (C-bands) was found to be near centromeric regions.

Radioactive RNA complementary to the H3 satellite of *H. vulgare* and the H1 satellite of *H. arizonicum* hybridize mainly in regions adjacent to centromeres. In addition to the centromeric locations of the H3 sequences, some chromosomes of the *H. vulgare* complement contain the sequence on other parts. Because of insufficient data, it is not possible at present to make detailed comparison between C-band pattern and the hybridization pattern of *H. vulgare*. Some chromosomes of *H. arizonicum* do not contain its H1 satellite sequences at all.

In situ hybridization studies which establish the locations of the satellite(s) sequences on chromosomes may therefore prove helpful in identifying chromosomes.

CHAPTER V

IN SITU HYBRIDIZATION STUDIES OF H3 (*H. VULGARE*) AND H1 (*H. ARIZONICUM*) SATELLITES WITH CHROMOSOMES OF OTHER *HORDEUM* SPECIES

INTRODUCTION

Studies with animal satellite DNAs indicate that some satellite sequences may be conserved in related species (Salser et al., 1976; Peacock et al., 1977a, 1977b; Dunsmuir, 1976; Lohe, 1977; Venolia, 1977; Appels and Peacock, 1978). Conservation of satellite sequences among plants has not been studied extensively. Analytical CsCl gradient analyses of DNAs from several species within the same genus have shown that the amounts of satellite at least, are not conserved (Beridze, 1972, 1975). Peacock et al. (1977a) have mentioned, though without providing details, that the satellite DNA of 'clipper barley' shares similar physical properties with one wheat satellite DNA and radioactive c-RNA made from it hybridizes with chromosomes of wheat. This observation led the authors to conclude that "a satellite sequence appears to have been stringently conserved during evolution". No information is available about conservation of satellite sequences among different plant species within a genus. In the previous Chapter it was shown that two of the three thermal components of satellite H1 of *H. arizonicum* have physical properties similar to those of the H3 satellite of *H. vulgare*.

This Chapter describes *in situ* hybridization studies involving the H1 satellite of *H. arizonicum* and the H3 satellite of *H. vulgare* with chromosomes of other *Hordeum* species.

MATERIALS AND METHODS

Materials

These studies were restricted because of lack of time to the combinations shown in Table 1.

Nonetheless the combinations used have given a general picture regarding conservation of these two satellite sequences among closely related and distantly related species within the genus *Hordeum*.

Sources of different *Hordeum* species have been listed in Chapter III.

Table 1

Species used for *in situ* hybridization

Satellite	Chromosomes of <i>Hordeum</i> species	
H3 (<i>H. vulgare</i>) representative of the diploid group.	* <i>H. spontaneum</i> (2x)	Members of the diploid group
	<i>H. agriocrithon</i> (2x)	
	<i>H. californicum</i> (2x)	Members of the poly- ploid series
	<i>H. arizonicum</i> (6x)	
H1 (<i>H. arizonicum</i>) representative of the polyploid series	<i>H. vulgare</i> (2x)	Members of the diploid group
	<i>H. spontaneum</i> (2x)	
	<i>H. agriocrithon</i> (2x)	
	<i>H. californicum</i> (2x)	Member of the poly- ploid group

METHODS

The procedures for H^3 c-RNA synthesis and *in situ* hybridization have been described in Chapter IV.

RESULTS AND DISCUSSION

(i) H3 Satellite Sequences of *H. vulgare*

The hybridization studies show that H3 sequences are present in the genomes of all four species tested (Figs. 1-4). All chromosomes of the three diploid species contain these sequences, although in various amounts and locations. One pair of chromosomes of *H. agriocrithon* (Fig. 1) and two pairs in the *H. californicum* complement have larger blocks of sequences than the other chromosomes. Although no quantitative estimations were attempted, hybridization of this satellite with *H. vulgare* appears to be more extensive (Fig. 13, Chapter IV) than with the other diploid species, which appear qualitatively similar to each other.

In contrast to the diploid species, some chromosomes of the hexaploid *H. arizonicum* are devoid of H3 sequences (Fig. 4). The others show discrete locations of these sequences. In one chromosome the location is near the secondary constriction region.

It is known that the *H. californicum* genome is present in the hexaploid *H. arizonicum*. All chromosomes of the former species show discrete locations of the satellite sequences whereas some chromosomes of the latter do not contain these sequences at all. It is concluded that chromosomes which lack the H3 sequences can not belong to the *H. californicum* complement and must come either from *H. compressum* (donor of one genome in the evolution of *H. arizonicum*,

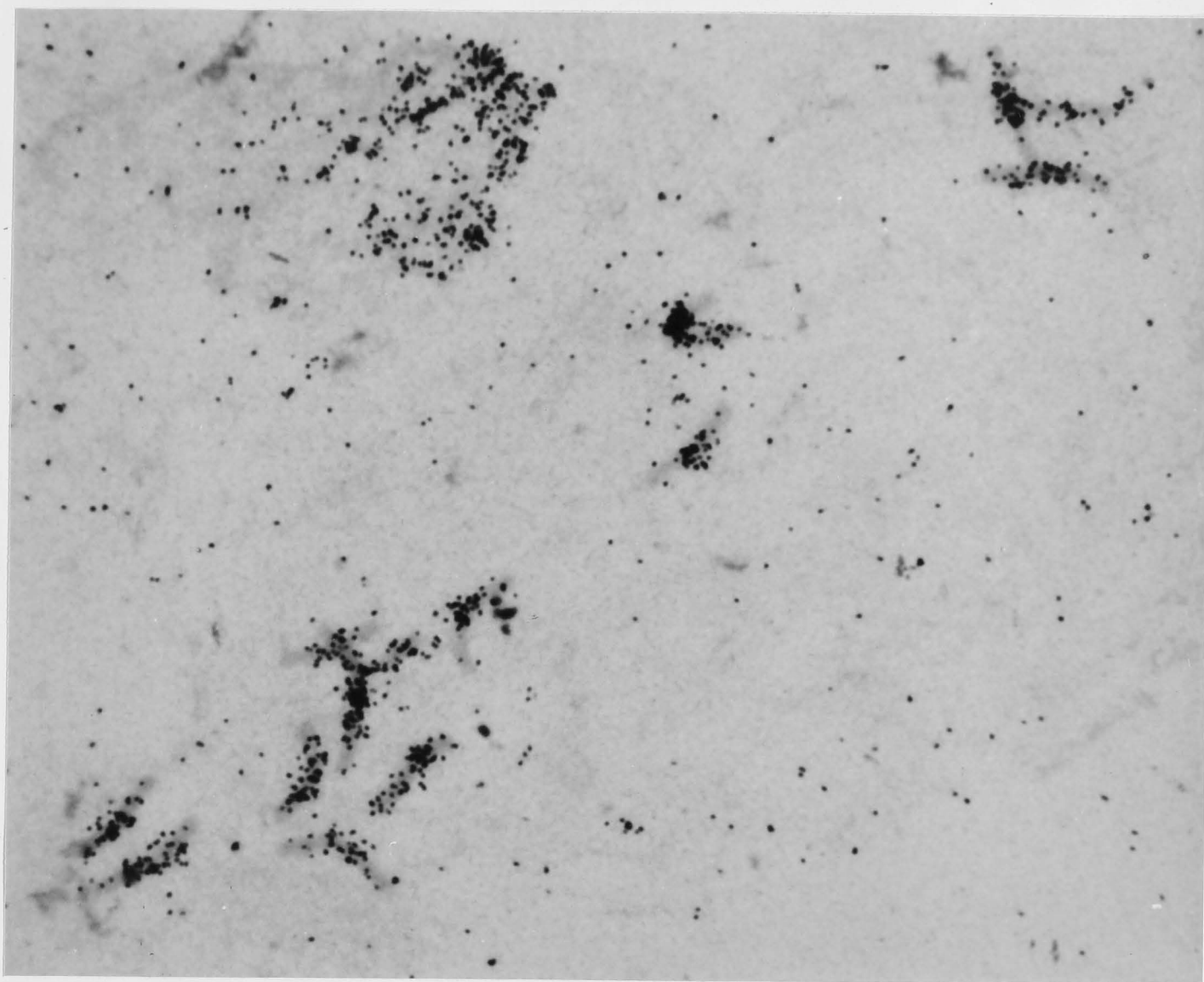


Fig. 1

Distribution of H3 (of *H. vulgare*) satellite sequences on *H. agriocrithon* ($2n = 2x = 14$) chromosomes. H^3C -RNA (7×10 cpm/ μ l) was used for *in situ* hybridization.

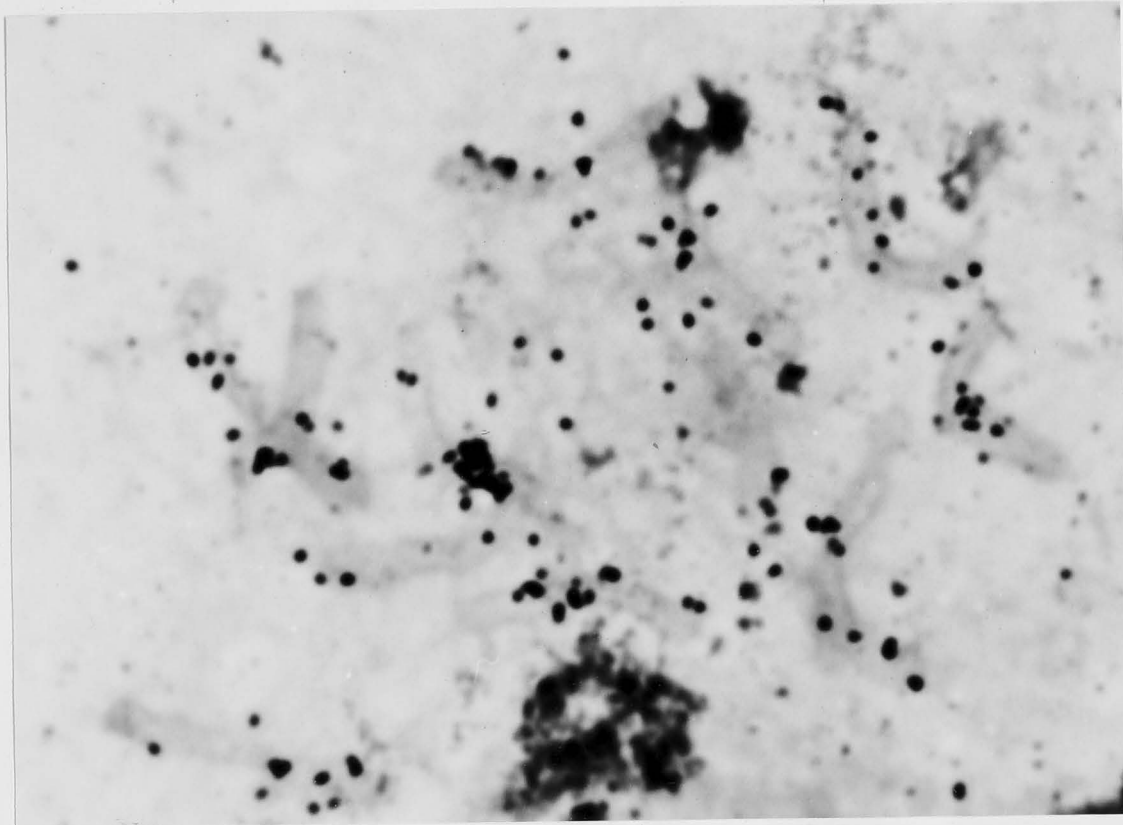


Fig. 2

Distribution of H3 (of *H. vulgare*) satellite sequences on *H. spontaneum* ($2n = 2x = 14$) chromosomes. H^3C -RNA (7×10^4 cpm/ μ l) was used for *in situ* hybridization.

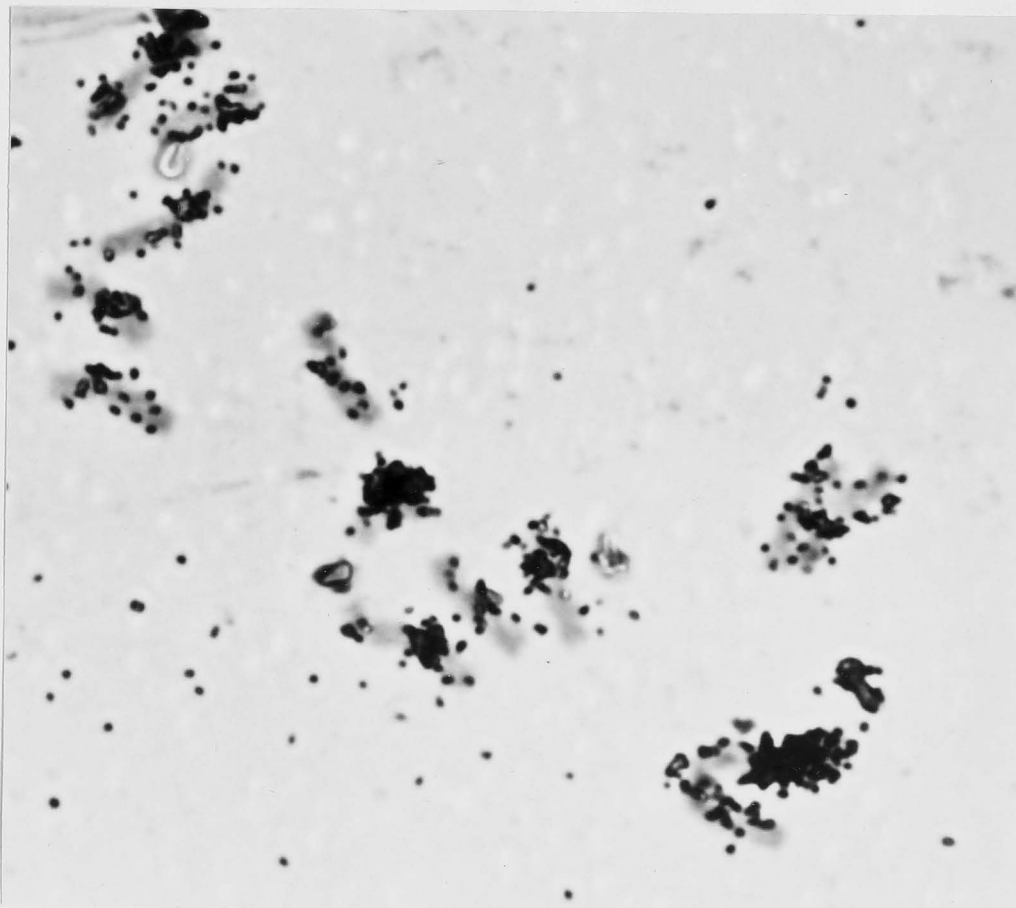


Fig. 3

Distribution of H³ (of *H. vulgare*) satellite sequences on *H. californicum* chromosomes ($2n = 2x = 14$). H³C-RNA (7×10^4 cpm/ μ l) was used for *in situ* hybridization.

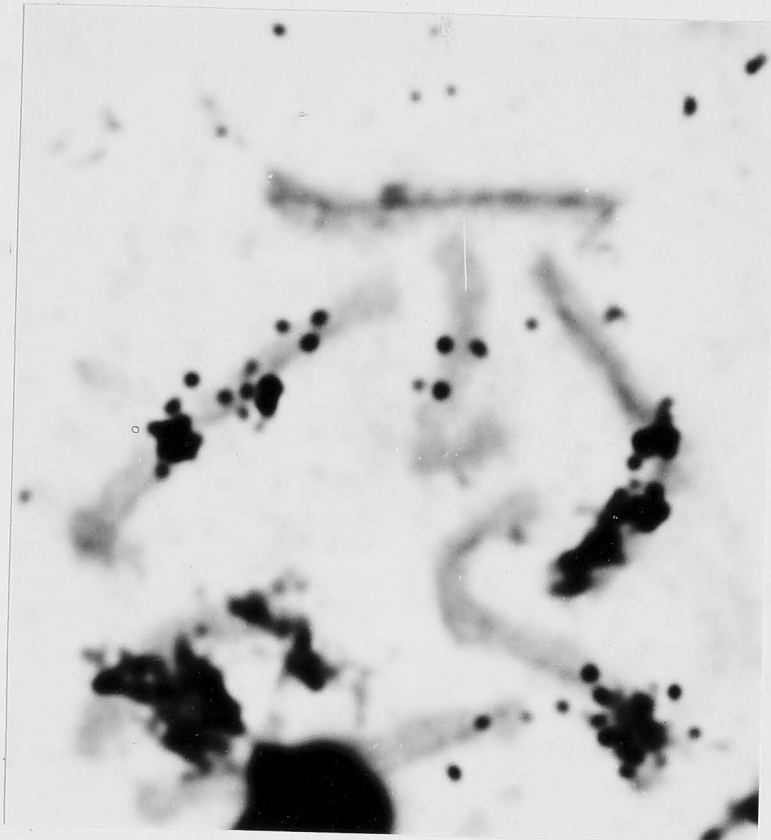


Fig. 4

Distribution of H3 (of *H. vulgare*) satellite sequences on *H. arizonicum* chromosomes. Not all the chromosomes are present in the Fig. H^3C -RNA (7×10^4 cpm/ μ l) was used for *in situ* hybridization.

Starks and Tai, 1974) or from the unknown donor, or from both.

(ii) H1 Satellite Sequences of *H. arizonicum*

Additional evidence to support the above conclusion comes from the *in situ* hybridization of H1 satellite (of *H. arizonicum*) with chromosomes of *H. californicum* (Fig. 5). The sequences of this satellite have discrete locations on all chromosomes (Fig. 5) of *H. californicum*, but not on all chromosomes of *H. arizonicum* (Fig. 20, Chapter IV). Further experiments with *H. compressum* (2x) and *H. jubatum* (4x), the two known genome donors in the evolution of *H. arizonicum* (6x), will be necessary to find out the source(s) of unlabelled chromosomes in the hexaploid species.

Hybridization of H1 satellite with chromosomes of *H. californicum* is more extensive than with the other diploid species, *H. vulgare*, *H. agriocrithon* and *H. spontaneum* (Fig. 6). This may mean that members of the diploid group have less H1 satellite sequences in their genomes. Alternatively, it may mean that although they have sequences similar to H1, the latter has some component which is not present in those three species and, as a result, extensive hybridization can not occur. Most probably the second possibility is the more likely. It has been shown (Chapter IV) that similar physical properties are shared by H3 satellite (*H. vulgare*) and two of the three thermal components of H1 satellite (*H. arizonicum*). It is presumably the presence of the third component, unique for H1 satellite, that interferes with its hybridization with genomes from which this component is absent (e.g. *H. vulgare*).

The H1 of *H. arizonicum* and H3 *vulgare* showed a single component in both $\text{Ag}^+ - \text{Cs}_2\text{SO}_4$ and CsCl gradients yet resolve multicomponents on thermal dissociation.

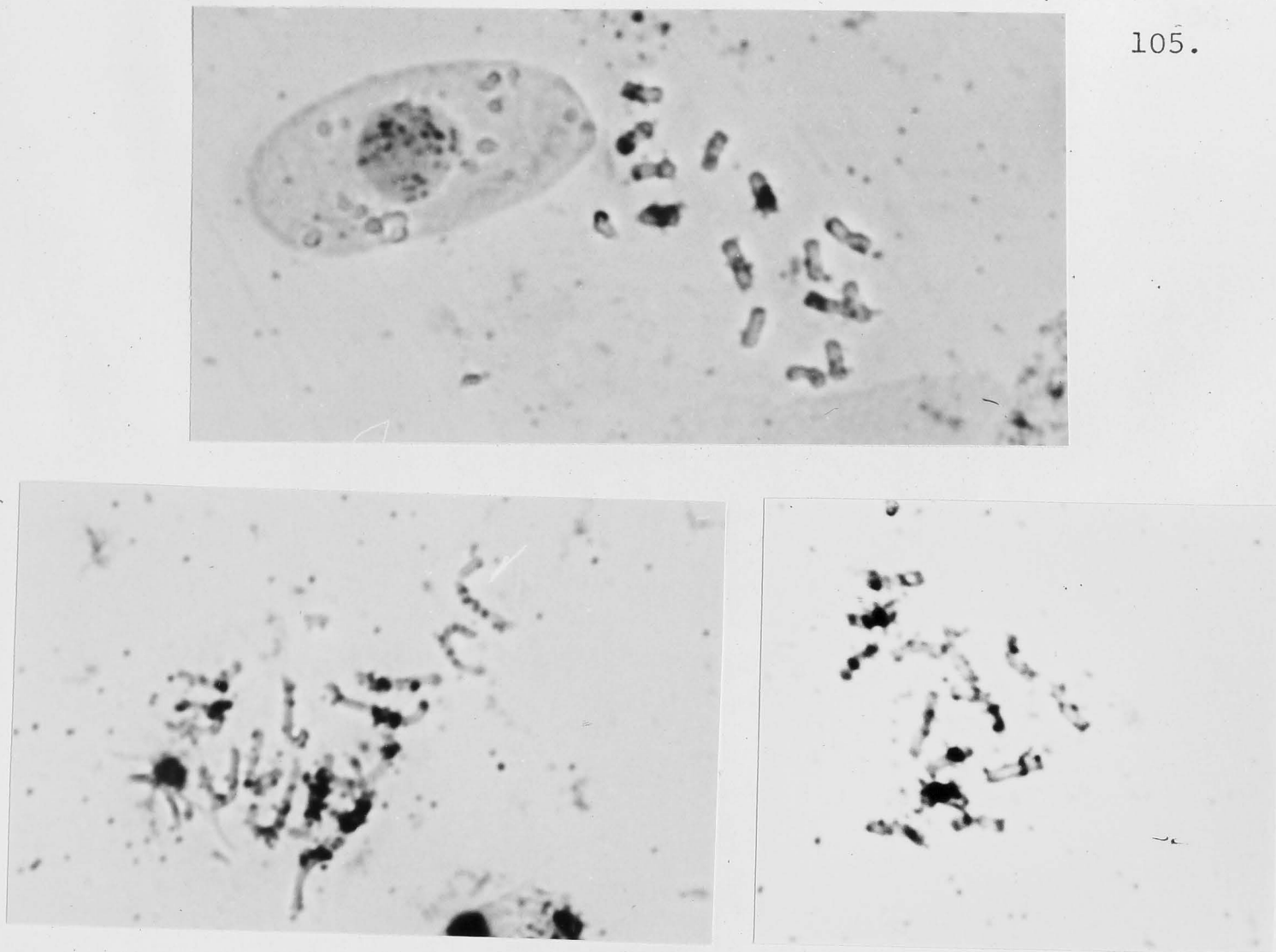


Fig. 5

Distribution of H₁ (of *H. arizonicum*) satellite sequences in the nuclei and chromosomes of *H. californicum* chromosomes ($2n = 2x = 14$). H³C-RNA (6×10^4 cpm/ μ l) was used for *in situ* hybridization.

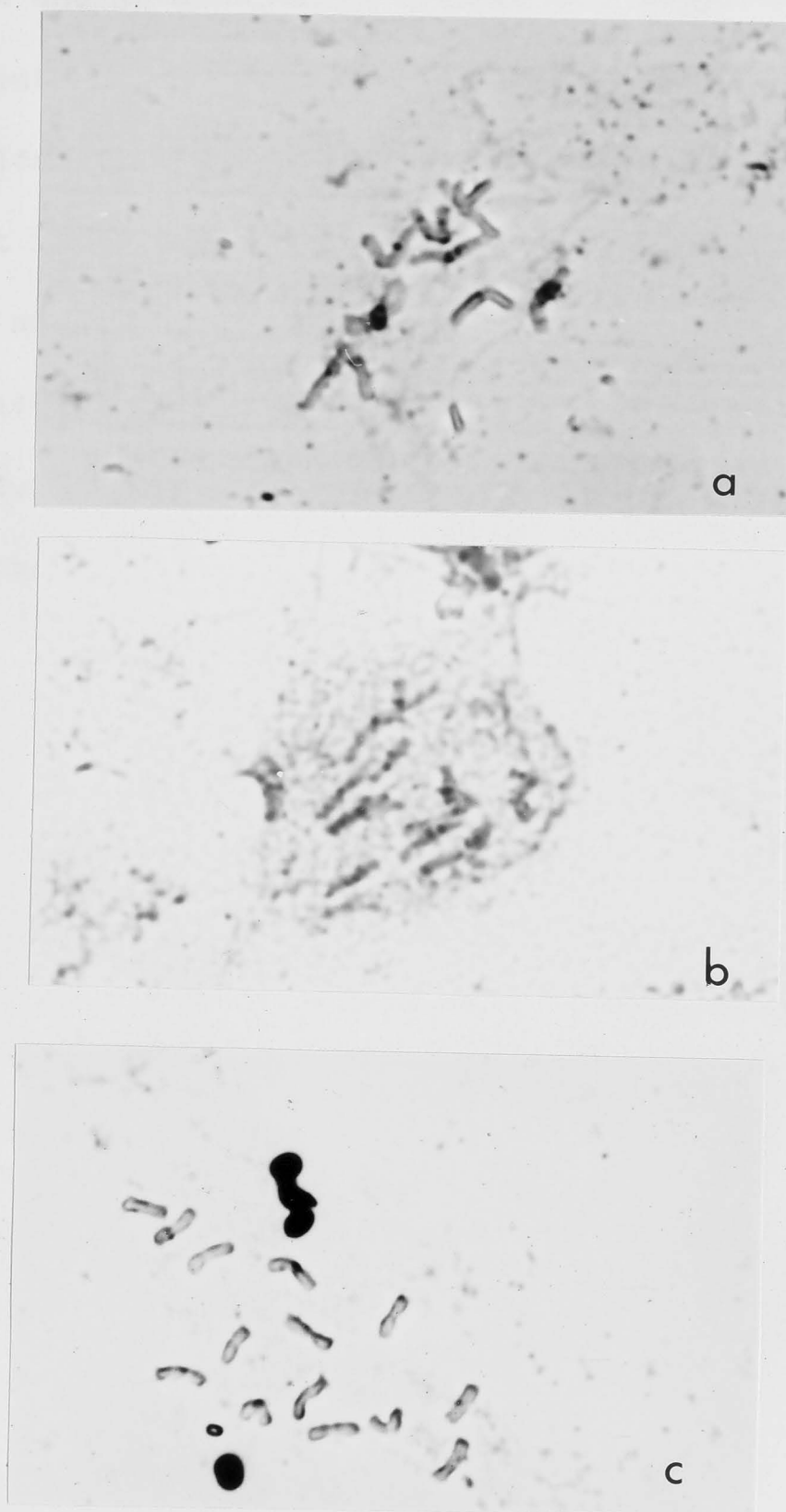


Fig. 6

Distribution of H1 (of *H. arizonicum*) satellite sequences on chromosomes of three diploid species of *Hordeum*. H^3 c-RNA (6×10^4 cpm/ μ l) was used for *in situ* hybridization.

(a) *H. agriocrithon*, (b) *H. vulgare*, (c) *H. spontaneum*.

Poor hybridization of the H1 of *H. arizonicum* with *H. vulgare*, *H. agriocrithon* and *H. spontaneum* chromosomes suggests that the thermal components in question are covalently bound.

The *in situ* hybridization studies demonstrate that sequences similar, if not identical, to those of H3 satellite of *H. vulgare* are present in all the *Hordeum* species studied.

Differential amounts and locations of the satellite sequences may prove helpful in chromosome identification and for genome analysis in situations like *H. arizonicum*.

CHAPTER VI

GENERAL DISCUSSION

This Chapter summarizes the principal conclusions of this thesis and where relevant these conclusions are discussed in relation to present-day dogma concerning the assumed properties of satellite DNA.

The six *Hordeum* species selected for this study fall naturally into two categories;

- (a) the diploid group of three closely related species (*H. spontaneum*, *H. agriocrithon* and *H. vulgare*);
- (b) the polyploid series consisting of three species, (*H. californicum* (2x), *H. jubatum* (4x) and *H. arizonicum* (6x), related to each other in having one genome in common.

H. vulgare and *H. arizonicum* were chosen for detailed analysis because they represent the terminal product of an evolutionary line in each of the two categories.

There are nine points which merit attention:

The influence of contaminating bacterial DNA on the analysis of plant DNA

The validity of the analyses carried out in this thesis depends on the purity of DNA isolated from the plants under examination. Several of the earlier claims for the occurrence of satellites in plant DNAs turned out to be due to contamination by bacterial DNA (Pearson and Ingle, 1972; Bendich, 1972; Sarrouy-Ballat et al., 1973; Broekaert and Parijs, 1975; Delseny, 1975). It was, therefore, necessary to test for the presence of such extraneous satellites and to effectively exclude them from the analysis. Several types of

bacteria were in fact found to be associated with *Hordeum* species. Silver nitrate treatment and aseptic germination of seeds proved effective in minimising bacterial flora from the seedlings of *H. vulgare*. As has been observed in other plants (Bendich, 1972), shoots of *Hordeum* harbour less bacteria than do roots. For this reason shoots were chosen as the source of DNA and it was found that AgNO_3 treatment could be omitted. Shoot extracts were routinely checked for the extent of bacterial contamination. The maximum contribution of bacterial DNA in the total DNA preparations was calculated to be 0.003% and at this level it does not interfere with the interpretation of the observed results.

It is possible that the same type of bacteria live in association with different species of *Hordeum* and also with *H. vulgare* grown in geographically different parts of the world. Whether this association has any significance for the biology of *Hordeum* is not known.

Similarities in DNA characteristics between species within the same genus

Buoyant densities of DNAs from six *Hordeum* species are very similar ($1.700\text{--}1.701 \pm 0.001 \text{ g/cm}^3$), so are their melting temperatures ($85.2\text{--}86.2 \text{ C}$). Similarities in these two characteristics reflect a similarity of overall base composition amongst the species concerned and the observation is consistent with the findings for other plant genera (Neurospora, Dutta et al., 1976; Zea, Levings, III et al., 1976; Allium, Ranjekar et al., 1978).

None of the six *Hordeum* species showed satellite DNA in a neutral CsCl gradient.

Organization of base sequences are different in two species

The amount of the repeated DNA fractions (reassociating up to a C_0t of 100) in *H. vulgare* is 70% which is probably not significantly different from *H. arizonicum* which has 65%. Ranjekar et al. (1978) did not find significant variations in the amount of repeated DNA in seven species of *Allium*.

The denatured DNA of *H. vulgare* follows kinetics of reassociation which is different from that of *H. arizonicum*, especially at low C_0t values. Corresponding low C_0t fraction DNAs of these two species differ in their physical properties and the DNA satellite patterns of these two species are also different. Moreover, the L2 satellite of *H. arizonicum* consists of two components (in CsCl) which have very low buoyant densities. Comparable low density components are not resolved from the DNA of *H. vulgare*.

All these results suggest that the base sequences of the repeated DNA fractions are arranged differently in the genomes of these two species.

This conclusion is, of course, based on observations from two distantly related species. It is not possible, at present, to say whether closely related species (e.g. *H. vulgare* and *H. spontaneum*) also show comparable differences in sequence organization.

Similarity of satellite pattern between related species

Species belonging to the diploid group (*H. vulgare*, *H. agriocrithon* and *H. spontaneum*) show similar, though not necessarily identical, patterns of cryptic satellites in $Ag^+-Cs_2SO_4$ gradients. Satellite patterns of members of the polyploid series (*H. californicum*, *H. jubatum* and *H. arizonicum*) are similar amongst themselves but are clearly distinguishable from the diploid group in having fewer heavy satellites.

The amount of satellite varies between species

Species showing similar satellite patterns vary in the amounts of satellites. Members of the polyploid series showed a decrease in the satellite amount with increase in ploidy. The progressive decrease in the satellite amount can be attributed either to dilution caused by the presence of additional genomes which lack or contain little satellite or else because each progenitor species has similar satellites for which modulation has resulted in reduced amounts when combined in a polyploid situation.

Further experiments with *H. compressum* (2x) and *H. jubatum* (4x), which are known to provide two genomic components in hexaploid *H. arizonicum*, are required to decide which of the two alternative explanations apply.

Variation in the amount of density satellite among different species is also found in *Phaseolus* and *Brassica* (Beridze, 1972, 1975 respectively). In *Brassica*, depending on the species, the amount varies from 0% to 37% of total DNA.

Satellite sequences most probably differ within a species

Different satellites within a species sometimes have similar base sequences (Gall and Atherton, 1974; Brutlag and Peacock, 1975; Endow, 1975) but in *Dipodomys* different satellites have different base sequences (Salser et al., 1976) (Table I).

All the *Hordeum* species studied have more than one satellite. Although sequencing was not attempted, there is good reason to think that satellites of different species of *Hordeum* are dissimilar in base sequences. The basis of resolution of satellite DNA in this study is the preferential binding of Ag^+ to fractions of DNA at alkaline pH. In *H. vulgare*, for example, three heavy and two light

satellites have been described. These two types of satellites are distributed in quite different densities in a $\text{Ag}^+ - \text{Cs}_2\text{SO}_4$ gradient and are separated by a colossal main band. All five satellites of this species were purified and each gave a single band in $\text{Ag}^+ - \text{Cs}_2\text{SO}_4$ gradients, yet light satellites showed the presence of two to four components in CsCl gradients. The *H. arizonicum* satellites behave similarly in buoyant density analyses. These observations suggest that light satellites are compositionally different from those of the heavy satellites.

Heterogeneity within a satellite

It is clear from the work of others that satellites homogeneous on one criterion may be shown to be heterogeneous when further tests are done. For example, some plant satellite DNAs look homogeneous in buoyant density analysis but their heterogeneity becomes evident in thermal denaturation profiles (melon, Bendich and Anderson, 1974; Bendich and Taylor, 1977; tomato, Chilton, 1975; cucumber, Timmis and Ingle, 1977). Other plant satellite DNAs (e.g. flax, Timmis and Ingle, 1977; and wheat, Ranjekar et al., 1978), do not reveal their cryptic heterogeneity until after reassociation studies.

A study of human satellite III by the combined techniques of restriction endonuclease digestion, gel electrophoresis and filter hybridization, has shown that the satellite judged homogeneous by the criterion of buoyant density may be "actually comprised of different populations of susceptible molecules each containing different sized elements and DNA base sequences, and each being unique to individual chromosome types" (Beuchamp et al., 1979).

In the present work none of the satellites (heavy or light) investigated in detail are homogeneous in base composition on all criteria. Heterogeneity was sometimes shown at the stage of analytical

ultracentrifugation in CsCl after seeming purification in $\text{Ag}^+ - \text{Cs}_2\text{SO}_4$ gradients (L1 and L2 of *H. vulgare* and L2 of *H. arizonicum*). All the others were homogeneous by buoyant density criteria but were evidently multicomponent when tested by the differential analysis of the thermal denaturation data and by thermal reassociation.

Are the satellites conserved?

The H1 satellite of *H. arizonicum* and the H3 of *H. vulgare* share some similar physical properties but differential analysis of denaturation data clearly shows that the two satellites cannot be identical. The presence of a third thermal component in H1 distinguishes it from the H3 satellite.

Radioactive RNA complementary to the H3 satellite of *H. vulgare* hybridizes with the chromosomes of four other *Hordeum* species, indicating the presence of similar, but not necessarily identical, sequences in all these species. This observation is not a convincing proof of conservation of the H3 sequences in *Hordeum*, since *in situ* hybridization allows a limited degree of cross-hybridization and subtle differences in satellite sequences would not be evident (Beuchamp *et al.*, 1979).

In this discussion, the conservation of satellite sequences among different species means that the nucleotide sequences concerned are absolutely identical. In this sense, it is not possible to say whether the H3 sequences are conserved in *Hordeum* species. The point can only be settled by sequence analysis.

Conservation of satellite sequences has been claimed for different organisms (Salser *et al.*, 1976; Peacock *et al.*, 1977a, b; Lohe, 1977; Mazrimas and Hatch, 1977; Venolia, 1978; Appels and Peacock, 1978). In most of the examples the claims were based on similar physical properties of the satellites and positive *in situ*

hybridization. As has been pointed out above, the techniques employed in these studies are not sensitive enough to detect subtle differences in the sequences concerned and therefore, these claims are premature. The sequences of only a few animal satellites are known (Table I). Most probably conservation is not a general phenomenon in eukaryotic genomes.

The molecular approach to phylogenetic studies

Results of the DNA analysis from all six *Hordeum* species agree well with the established taxonomic relationship among them. One group of *Hordeum* species can be distinguished from the other group by the characteristics of the highly repeated DNAs. Although minor differences exist, members within each group have similar patterns of organization of the satellite DNA fractions in their genomes.

Although no comparable DNA analysis data are available for species within another genus, Flavell *et al.* (1977) have studied the highly repeated DNA fractions from four cereal plants of the tribe *Triticineae*. They concluded that the amount of repeated fractions held in common diminishes with taxonomic separation of the species.

There is no *a priori* reason why the molecular approach to phylogenetic study should agree with the taxonomic classification since the former utilized mainly the highly repeated DNA sequences which, at present, are thought to have no genic functions. The taxonomists, on the other hand, utilize phenotypic characters which are encoded in the unique sequences of the genome. In order to specify phenotypic differences between two species, some of the coding sequences in one species must be different from another.

Results of this thesis and findings of Flavell *et al.* (1977) suggest that the highly repeated sequences are in some way associated with the speciation events.

TABLE I

Satellite sequences of some animal DNA (modified from John and Miklos, 1979)

Species	Satellite	Sequence	Reference
<i>Drosophila melanogaster</i>	1.672	AATAT	Peacock et al. 1973, 1977
	1.672	AATATAT	Endow et al., 1975
	1.686	AATAACATAG and variants	Sederoff et al., 1975 Manteuil et al., 1975
	1.688	Complex and variants	
	1.705	AAGAG	Shen and Hearst, 1977
	1.705	AAGAGAG	Brutlag et al., 1977a,b
<i>D. virilis</i>	I	ACAAACT	Gall and Atherton, 1974
	II	ATAAACT	
	III	ACAAATT	
Kangaroo rat	MS	$\begin{array}{cc} \text{A} & \text{AG} \\ \diagdown & \\ \text{G} & \text{C} & \text{A} \end{array}$	Salser et al., 1976
	HS- α	$\begin{array}{ccccccc} \text{G} & \text{G} & \text{G} & & \text{T} & \text{T} & \text{A} \\ & & \diagdown & & & & \\ & \text{A} & \text{A} & \text{T} & & \text{G} & \end{array}$	
	HS- β	ACACAG C GGG	Fry et al., 1973 Marx and Hearst, 1975
		$\begin{array}{ccc} & \diagdown & \\ & \text{A} & \text{G} & \text{G4 or} \\ & & & \text{G5} \end{array}$	
Guinea pig	$\alpha = \text{I}$	GGGTTA and variants	Southern, 1970
Mouse	Mouse satellite	GAAAAATGA	Southern, 1975; Biro et al., 1975; Horz and Zachau, 1977; Maio et al., 1977

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